REPORT

TECHNICAL REPORT AND TESTING PROTOCOL FOR COMMERCIAL MICROBIOLOGICAL AMENDMENT TESTING AND EVALUATION

To

Air Force Center for Environmental Excellence

Brooks AFB, TX 78235

December 1, 1995

Battelle
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TECHNICAL REPORT AND TESTING PROTOCOL

FOR

COMMERCIAL MICROBIOLOGICAL AMENDMENT TESTING AND EVALUATION

to

Air Force Center for Environmental Excellence
8005 9th Street
Brooks Air Force Base, Texas 78235

by

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

1.0 INTRODUCTION

Microbiological degradation of various pollutants has been studied intensively within the past 15 years. It was established early on in laboratory studies that petroleum hydrocarbon compounds are particularly susceptible to microbial degradation. The Air Force has identified more than 1,300 JP-4 jet fuel spill sites under the Installation Restoration Program. The majority of these sites have high levels of fuel absorbed or occluded in the soils. When a fuel spill occurs, a major portion of the fuel is adsorbed to the soil matrix. The fuel then slowly releases water-soluble compounds, such as benzene, into the groundwater. Given the large number of areas contaminated with petroleum hydrocarbons, bioremediation would appear to be a promising cost-effective and efficient technology to use at these sites. Commercial microbial amendments typically are promoted for use at these sites, when often indigenous microorganisms are present and are capable of degrading the contaminants.

Numerous vendors offer for sale and promote the use of proprietary microbial mixtures for in situ bioremediation of contaminated sites. Often, these vendors have a significant quantity of laboratory and field data demonstrating the effectiveness of their formulas. However, the use of proper controls generally is not observed, making it difficult to judge the true usefulness or cost-effectiveness of a microbial amendment, or to accurately compare amendments with each other or with indigenous microorganisms. Vendors of commercial microbial amendments often minimize the ability of indigenous microorganisms to degrade the same contaminants as microbial amendments at much lower cost. To Battelle's knowledge, studies published in the peer-reviewed literature have not supported many of the vendor claims, and call into question the real advantage of microbial addition (Atlas, 1991). Based on Battelle's experience with in situ bioremediation, biodegradation of petroleum hydrocarbons is limited not by the lack of indigenous bacteria capable of degrading the contaminants, but by the oxygen supply which is used up rapidly by the bacteria during degradation of the hydrocarbons. However, it is possible that situations exist where microbial amendments significantly enhance the degradation of certain contaminants under certain conditions.

Although many studies have documented biodegradation of petroleum hydrocarbons, both with commercial microbial amendments and with indigenous microorganisms, there is no laboratory protocol for comparing the biodegradative capability of each source of microorganisms when tested under identical conditions for in situ applications. Proponents exist for both the commercial and the indigenous method of bioremediation, and convincing arguments based on laboratory and field studies can be made for either side; however, unless these tests are conducted under identical conditions, it is difficult to compare the data and evaluate the technologies. In addition, it would be impossible and impractical for each Air Force Base to

attempt to evaluate each new commercial microbial amendment entering the marketplace. A more cost-effective approach is to develop a standard laboratory test that can be applied to evaluate new commercial microbial amendments of interest. The purpose of the work described in this document is to develop this test.

The laboratory testing protocol was designed to determine quantitative differences based on biodegradation rates of commercial microbial amendments and indigenous microorganisms. The evaluation protocol will include sufficient controls to determine whether any enhancement is due to the addition of bacteria, or nutrients included in the amendment product, or both. The protocol will involve laboratory studies that compare, under ideal conditions, biodegradation of petroleum hydrocarbons by both commercial microbial amendments and indigenous microorganisms from contaminated sites. Development of an objective testing and evaluation (T&E) program would assist Air Force Bases in selection of remediation technologies by eliminating unnecessary addition of commercial microbial amendments or by determining which types of sites may benefit most from the addition of commercial microbial amendments, potentially saving money by eliminating ineffective treatments.

Parameters such as soil type and temperature may have a significant effect on biodegradation of petroleum hydrocarbons. It is possible that the addition of commercial microbial amendments may be more feasible at sites with certain soil characteristics. Comparison of biodegradation rates in different soil types such as clays, silts, or sands would elucidate whether commercial microbial amendments are more practical in certain soils. In addition, soil temperature has a significant effect on microbial metabolism. For example, although commercial microbial amendments may function well in temperate climates, use of the amendments in subarctic or tropical climates may be impractical. Conducting biodegradation studies under different temperatures would help to identify those climates where addition of commercial microbial amendments is more or less suitable.

This report contains descriptions of the experiments that were conducted during the development of a testing and evaluating protocol that can be used by the Air Force for screening microbial amendments. The experiments were selected to determine if amendments enhance degradation as well as to determine whether added bacteria or nutrients are responsible for any enhancement. Analyses of soil properties such as pH, particle size distribution, and cation exchange capacity were included in the protocol. Soils were analyzed for nutrients at the beginning and end of each experiment. Microbial activity was monitored through use of respiration measurements, dehydrogenase activity, and enumeration. Degradation performance was measured based on a mass balance for petroleum hydrocarbons.

Petroleum hydrocarbon analysis was performed on gas samples collected whenever the atmospheres in the column reactors were exchanged, and in soil samples collected during reactor setup and at the end of 30

Table 1. List of Specific Petroleum Hydrocarbon Compounds Quantified in Gas and Soil Samples.

Compound Name	Chemical Formula
isopentane	$CH_3CH_2CH(CH_3)_2$
n-pentane	$CH_3CH_2CH_2CH_2CH_3$
2-methyl-pentane	$CH_3CH_2CH_2CH(CH_3)_2$
n-hexane	$CH_3(CH_2)_4CH_3$
2,4-dimethyl-pentane	$(CH_3)_2CHCH_2CH(CH_3)_2$
benzene	C_6H_6
n-heptane	$CH_3(CH_2)_5CH_3$
toluene	$C_6H_5CH_3$
n-octane	$CH_3(CH_2)_6CH_3$
ethylbenzene	$C_6H_5CH_2CH_3$
p-xylene	$C_6H_4(CH_3)_2$
o-xylene	$C_6H_4(CH_3)_2$
n-propylbenzene	$CH_3CH_2CH_2C_6H_5$
n-decane	$CH_3(CH_2)_8CH_3$
n-butylbenzene	$CH_3CH_2CH_2CH_2C_6H_5$
n-dodecane	$CH_3(CH_2)_{10}CH_3$
n-tridecane	$CH_3(CH_2)_{11}CH_3$
n-tetradecane	$CH_3(CH_2)_{12}CH_3$
n-pentadecane	$CH_3(CH_2)_{13}CH_3$

days of incubation. Gas chromatographic analysis was used to identify and quantify the 19 compounds listed in Table 1 and to quantify boiling point splits based on hexane. Total petroleum hydrocarbons (TPH) were determined for each sample. The data were used to quantify the effectiveness of contaminant removal for the different experimental conditions.

2.0 TEST OBJECTIVES

The objective of the work described in this report was to develop a testing and evaluation protocol for use by the Air Force to screen commercially available microbial amendments for remediation of petroleum hydrocarbon-contaminated soils. The objective of the protocol is to demonstrate, under carefully controlled conditions, whether these amendments provide a significant improvement over indigenous microorganisms in biodegradation of petroleum hydrocarbons. The results of the tests outlined in the protocol are to assist staff in selecting technologies which will provide the most efficient and cost-effective solutions to their remediation needs. Screening out ineffective microbial preparations prior to field-scale application would result in significant cost savings for the Air Force. Conducting the tests contained in the protocol will provide a standardized procedure for collecting the data necessary to screen amendments that are marketed as remedies for hydrocarbon-contaminated soils.

3.0 EXPERIMENTAL DESIGN

Because petroleum hydrocarbons are degraded predominantly by aerobic means, all laboratory studies were conducted under aerobic conditions. If commercial microbial amendments specified for aerobic treatment of petroleum hydrocarbons were to be added to an oxygen-limited site without addition of oxygen, they most likely would fail in the goal to promote bioremediation.

Four factors were considered in designing the laboratory test apparatus and selecting the analytical methods during the development of the testing protocol:

1. The ability to conduct tests using different soil types at different temperatures.
2. Conducting tests under conditions representative of in situ conditions.
3. Providing adequate controls to fully evaluate any enhancements.
4. Collecting data that allow for the calculation of biodegradation rates.

Three runs of degradation experiments were conducted in column type and/or biometer flask reactors. Five experimental conditions were examined in the initial experiment, and the number of conditions in the two subsequent runs were adjusted according to the results obtained from the preceding runs.

To develop a smoothly operating procedure, all of the developmental experiments were conducted using a single commercial microbial amendment identified as WMI-2000. The amendment was provided at no charge by Waste Microbes, Inc., of Houston, Texas, and a single soil type was collected from site

Table 2. Experimental Conditions for Each Column Reactor Type.

Column ID	Experimental Conditions				
	Uncontaminated Soil	Contaminated Soil	Jet Fuel	Live Amendment	Sterilized Amendment
Control 1	×				
Control 2		×			×
Reactor 1		×			
Reactor 2	×		×	×	
Reactor 3		×		×	

POL-B at Tyndall Air Force Base (AFB), Florida. Battelle had conducted several site investigations at POL-B that indicated that the soil would serve the purposes of this study very well. The soil was a fine sand, and both contaminated (< 500 mg/kg TPH) and uncontaminated soils were collected from this site.

Soil was collected using a brass sleeved split-spoon sampler technique. Soil gas probes were driven into the ground, and soil gas was extracted and analyzed for TPH concentration using a TraceTechtor TPH analyzer. A hot spot (> 10,000 ppm TPH) was located, and the spoon was driven and the sleeves retrieved. The sleeves were capped, labeled, put in a cooler on ice, and sent back to Battelle's laboratory in Columbus, Ohio. Uncontaminated soil was collected from a site not registering TPH in the soil gas using the same sleeved spoon technique.

The following sections contain the procedures that were used to prepare the soils for the laboratory experiments described in this report, to prepare the column and/or biometer flask reactors, and to monitor and assess the biodegradation performance under each experimental condition. The data were collected and evaluated to determine which reactor configuration, experimental conditions, and analytical protocols should be included in the final testing and evaluation protocol. The experimental methods, analytical methods, results, and conclusions are presented by experiment number and in experimental order in Sections 4.0 through 6.0 to assist in following the protocol development process.

4.0 EXPERIMENT #1

The first round experiments were conducted following the methods as described in the Experimental Design Test Plan (Battelle, 1994). Reactors were established in triplicate under the five different experimental conditions shown in Table 2. The experimental design included two controls. The first control contained only uncontaminated soil to provide data on the background respiration rate. The other control contained contaminated soil and a sterilized solution of the commercial microbial amendment to provide data on the effects of sample handling and nutrient and moisture addition from the addition of the amendment. The commercial microbial amendment was filter-sterilized to minimize its effect on the chemical composition of the solution. The remaining three column reactors contained (1) uncontaminated soil, jet fuel, and an inoculum of the commercial microbial amendment; (2) contaminated soil and no amendment; and (3) contaminated soil and an inoculum of the commercial microbial amendment.

Establishing reactors under the five conditions described above allowed a distinction to be made between biodegradation due to activity from commercial microbial amendment and/or biodegradation due to indigenous microbial activity. In addition, this experimental design accounted for any natural background activity or any biodegradation enhancement from nutrients or other chemical compounds contained in the commercial microbial amendment product. The following sections contain descriptions of the soil processing method and the reactor design and setup, reactor operation and monitoring, and reactor harvesting procedures used for Experiment #1.

4.1 Experimental Methods

The text contained in the following sections describes the methods used during the first experiment to process the soils, set up the column reactors, operate and monitor the reactors, and harvest the reactors at the end of this experimental run. All of the methods described in these sections are based directly on the methods as described in the Experimental Design Test Plan (Battelle, 1994).

4.1.1 Soil Processing

Prior to setting up the column reactors, it was necessary to homogenize batches of contaminated and uncontaminated soil large enough to fill all of the required columns. Both the uncontaminated soil and contaminated soil were homogenized by hand kneading in a sealed plastic Ziploc™ freezer bag (2-gallon

size). The preliminary moisture content of the contaminated and uncontaminated soils was measured to determine how much distilled water or amendment must be added to make the moisture content 20%. Baseline values for the TPH in the uncontaminated soil were obtained through the TPH analysis described in Section 4.2.3. The homogeneity of the contaminated soils was determined based on TPH analysis of sequential samples from the freezer bag. When three subsequent samples fell within 10% of each other, the kneading process was terminated and then approximately 600 g of contaminated or uncontaminated soil were transferred from the Ziploc™ freezer bags into each of five plastic bags. The appropriate soil amendments were added (see Table 2), the moisture was adjusted to 20%, the bags were sealed, and the soils were hand-kneaded for 1.5 hours to ensure homogeneity.

4.1.2 Reactor Design and Setup

Column-type reactors were configured using 25 × 150-mm glass chromatography columns purchased from the Supelco Company. These reactors were used to conduct experiments to evaluate in situ microbial amendments. Figure 1 contains a schematic of the design for the reactors that were used. All components used in the construction of the column reactors were readily available from scientific suppliers.

Fifteen reactors were established for the protocol development experiments, including triplicate reactors for each of the five experimental conditions listed in Table 2. Each reactor was filled with 87 g of wet soil from the bags prepared as described in Section 4.1.1. The end caps of the reactors were secured in place and the columns were mounted to a floor rack. The water jackets from the reactors were connected using Tygon™ tubing so that all of the reactors were in series. The tubing was plumbed to a temperature-controlled circulating water bath capable of both heating and cooling.

During the protocol development experiments, the temperature was held at 25 °C. Although soils usually are not maintained at 25 °C in the field, this temperature is optimum for the laboratory evaluation of the various assays and analytical protocols. It will be specified in the finalized protocol that the soil temperature should be maintained within the temperature range specified by the vendor for the effectiveness of a specific amendment product, or at the temperature at the Air Force facility where the amendment would be applied.

The reactors in series were incubated for a period of 60 days. The atmosphere of each reactor was exchanged routinely throughout the incubation period with the reactors kept in place. After the 60-day incubation period, the reactors were removed from the floor rack and harvested for the analyses on the incubated soils.

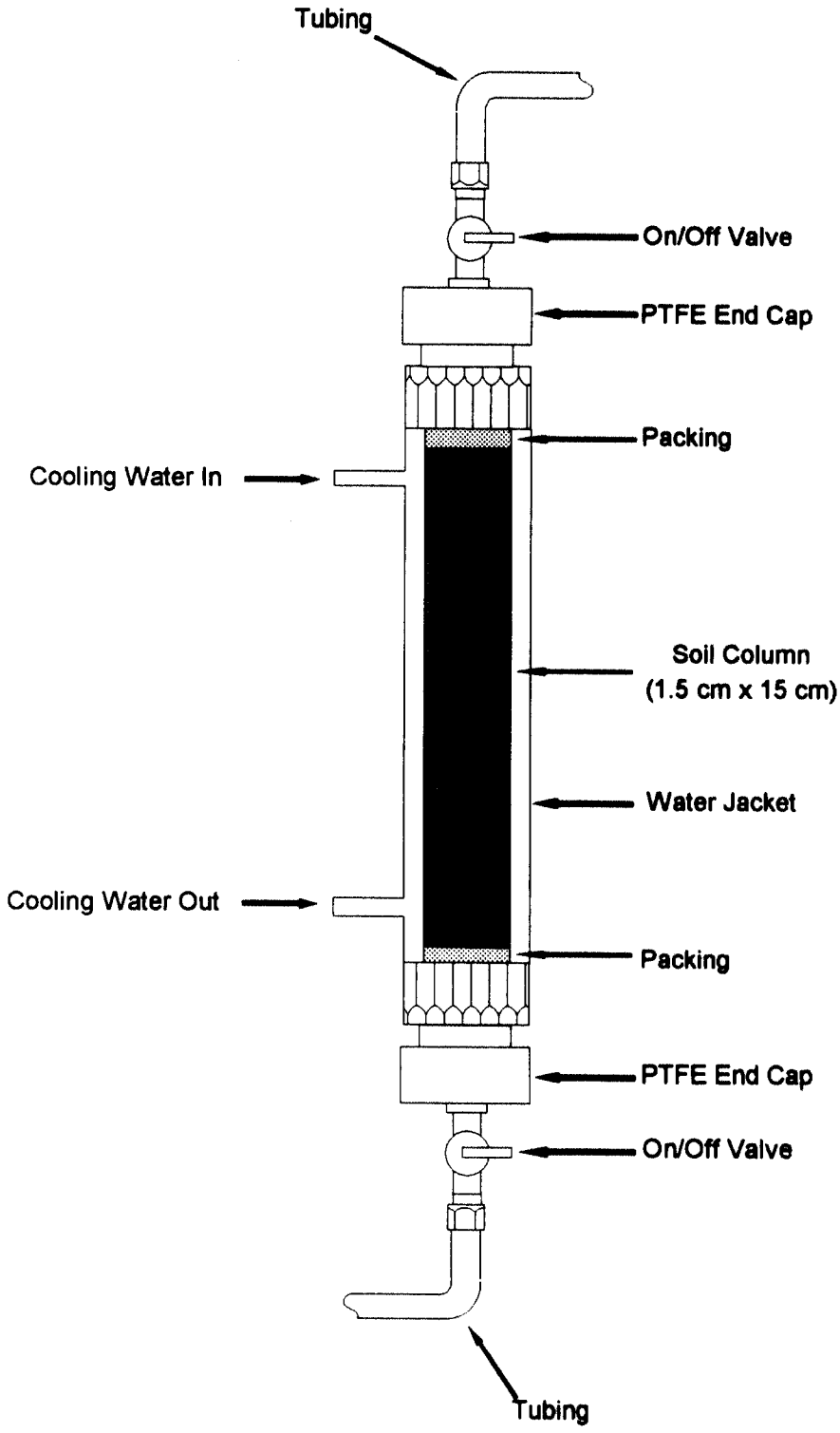

Figure 1. Design Schematic for the Column Reactors to be Used to Test Commercial Microbiological Amendments (drawing not to scale).

4.1.3 Reactor Operation and Monitoring

4.1.3.1 Maintaining Temperature. The use of the jacketed column reactors allowed accurate control of reactor temperatures. The reactor jackets were connected in series and plumbed to a circulating temperature bath that was set and maintained at 25 °C. The water in the return line was monitored beyond the place where water exited the last reactor in the series to ensure that the temperature was being maintained. The water in the bath was exchanged as needed.

4.1.3.2 Atmosphere Exchanges. To maintain an aerobic environment in each column reactor, it was necessary to exchange the reactor gas on a regular basis. The amount of time between gas exchanges was determined based on oxygen measurements made by direct withdrawal and gas chromatographic (GC) analysis using a gastight syringe. For this experimental period, samples usually were taken every 7 days, but varied depending on the measurements of oxygen. The gas was exchanged using the apparatus shown in Figure 2. First, a clean Tedlar™ bag was filled with 200 mL of lab air and fastened to the bottom of the column being sampled. The Tedlar™ bags collecting the column air samples were flushed twice with ultrahigh-purity helium, using the desiccator to expel the helium. This method of flushing the sample bags clean proved to be more of a hassle than a benefit. After the first sampling process was over, the sampling bags were flushed twice with ultrahigh-purity helium directly from the helium tank. The sampling Tedlar™ bags were attached to the influent line in the lid of the desiccator and then opened (valve one on desiccator is closed). The lid was placed onto the desiccator and a 400-mL vacuum was pulled on the desiccator by using a 1000-mL syringe. The desiccator was then connected to the top of the column to be sampled. Valve one of the desiccator was opened, followed by the top valve on the column, and then the bottom valve on the column. The valve on the clean air Tedlar™ bag was opened slowly to allow the lab air to push the column air into the sample bag. When the clean air bag was empty, the valve on the bag was closed, followed by the bottom column valve, and then the top column valve. Valve one on the desiccator was closed. Valve two on the desiccator was opened to allow the rest of the vacuum on the desiccator to be released. The desiccator and valve on the Tedlar™ bag were closed so that none of the sample would be lost. The sample bag was pulled off of the influent line of the desiccator lid. Then the oxygen, carbon dioxide, and hydrocarbon contaminant concentrations of the sample in the Tedlar™ bag were analyzed according to the directions in Sections 4.2.1 and 4.2.2. Between each sample, the desiccator tubing was flushed with 800 mL of lab air to avoid possible sample cross-contamination. The uncontaminated soil with amendment and jet fuel addition

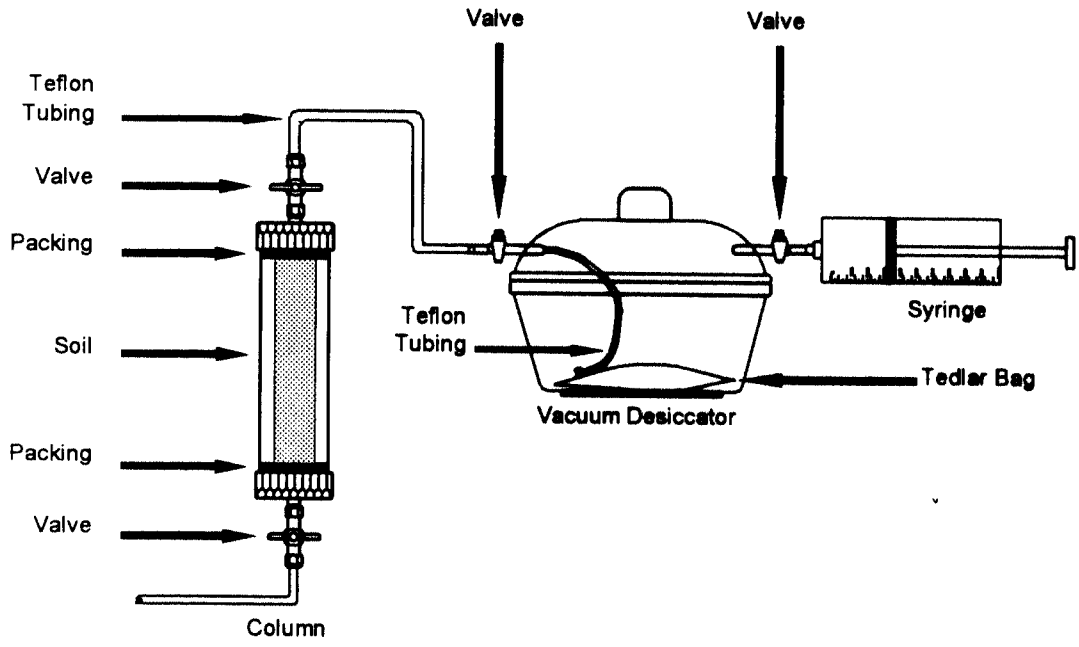

Figure 2. Schematic of the Sampling Apparatus used to Exchange Atmospheres in Column Reactors During Experiment #1.

was always sampled last to minimize cross-contamination because that condition contained the highest TPH concentrations.

On the fifth sampling series, two 100-mL samples were taken from the uncontaminated soil plus amendment and jet fuel to test whether the second 100-mL sample had the same oxygen concentration as the first 100-mL sample. This test was performed to minimize possible diluting of the samples. In the preceding nine sampling series, 100 mL of air was exchanged instead of 200 mL.

4.1.3.3 Monitoring Respiration. Oxygen and carbon dioxide concentrations were measured in the gas collected from each of the atmosphere exchanges. The data were used to monitor the oxygen concentrations to ensure that the oxygen levels had not become rate-limiting and to determine biodegradation rates. The final evaluation of biodegradation performance under each of the experimental conditions was assessed using data collected from analyses of initial and final soil samples. The samples were analyzed for oxygen, carbon dioxide, and petroleum hydrocarbons according to the methods described in Sections 4.2.1 and 4.2.2.

Initial soil samples were obtained directly from the homogenized batch of soil at the time of reactor setup. The final soil samples were collected from each of the reactors under each experimental condition on the 60th day following setup. The soil samples were analyzed for petroleum hydrocarbons, soil moisture, soil pH, nutrient concentrations, dehydrogenase activity, and microbial enumeration according to the methods described in Section 4.2.

4.1.4 Reactor Harvesting

After the 60-day incubation period it was necessary to remove the mounted column reactors from the floor rack in order to conduct further testing. The water cooling system was shut off and disconnected from the reactor assembly, and the Tygon™ tubing was removed from each water jacket. The water remaining within the water-jacket assembly was drained through a opening at the barbed fitting. When all of the water was removed so that no water would interfere with the soil in the columns, one of the end caps of the column being harvested was loosened and removed.

Each reactor was disassembled individually. This was done to ensure that there would be no cross-contamination of samples between reactors and to limit moisture and TPH loss during harvesting. A clean piece of wax paper was used under each reactor and discarded after the soil was removed. A clean stainless steel spatula was used to transfer soil to its respective sample vial or bottle. Spatulas were cleaned with tap water and flamed over a Bunsen burner between each reactor harvest to eliminate any carryover of soil

constituents and microorganisms between samples. Soil transfers were made into clean acid-washed bottles and vials with Teflon™-lined caps, where they were held until analyses were performed. All transfer bottles, tubes, and vials were prelabeled to facilitate the harvesting process, and each reactor was harvested in an identical manner to ensure consistency during sample collection.

After the cap was removed from the reactor, the top 3 cm of soil was transferred into a clean I-Chem® bottle for sample archiving. The next few grams of soil were removed quickly and transferred to vials to conduct soil total organic carbon (TOC) and TPH analyses. The vials were filled to the top to limit the amount of headspace and loss of hydrocarbon by volatilization. The next several grams of soil were removed and placed into a preweighed aluminum pan for moisture analysis. The wet soil and pan were weighed immediately to eliminate any error associated with rapid moisture loss. The next few grams of soil were removed for triplicate aliquots to be used in the microbial enumeration analyses. Each aliquot of soil was dispensed into a sterile dilution test tube which had been pre-tared on the scale. Approximately 1 g of wet soil was placed into each test tube. The test tubes were immediately capped and placed into the test tube rack for further dilution. After these soils were collected, a 10 g aliquot of wet soil was removed and placed into a preweighed, labeled 20-mL glass scintillation vial for the measurement of dehydrogenase activity. Finally, approximately 5 g of soil was removed and placed into a glass beaker for pH analysis. The remaining wet soil was placed into I-Chem® bottles. One of the bottles was transferred to a freezer where it was held at a temperature of –20°C for archiving. This temperature was necessary to ensure that most of the microbial activity would be inhibited. The other bottle was sent to an outside laboratory for nutrient analyses.

4.2 Analytical Methods

The soil and exchanged gas from each reactor during this experiment were analyzed for each of the analytes listed in Table 3. The analytical methods employed to measure each of the listed analytes are described in the following sections. Each of these methods is based directly on the methods described in the Experimental Design Test Plan submitted in December 1994 (Battelle, 1994).

4.2.1 Oxygen and Carbon Dioxide in Gas Samples

Oxygen and carbon dioxide were measured in the gas collected from each reactor during the atmosphere exchanges. The data were used to monitor the oxygen concentrations to ensure that the oxygen levels had not become rate limiting, and to determine the biodegradation rates over the period of incubation.

Table 3. List of Analytes and Analytical Schedule for Experiment #1.

Analysis	Soil					Gas				
	Column Reactor ID					Column Reactor ID				
	1	2	3	4	5	1	2	3	4	5
Oxygen	-	-	-	-	-	5	5	5	5	5
Carbon Dioxide	-	-	-	-	-	5	5	5	5	5
Petroleum Hydrocarbon	I,F	I,F	I,F	I,F	I,F	5	5	5	5	5
Moisture Content	I,F	I,F	I,F	I,F	I,F	-	-	-	-	-
pH	I,F	I,F	I,F	I,F	I,F	-	-	-	-	-
Dehydrogenase Activity	I,F	I,F	I,F	I,F	I,F	-	-	-	-	-
Microbial Enumeration	I,F	I,F	I,F	I,F	I,F	-	-	-	-	-
Nutrient Concentration	I,F	I,F	I,F	I,F	I,F	-	-	-	-	-
Carbon Content	I,F	I,F	I,F	I,F	I,F	-	-	-	-	-
Particle Size Distribution	I	-	-	-	-	-	-	-	-	-
Cation Exchange Capacity	I	I	I	I	I	-	-	-	-	-

Codes: I - Initial Sample, Only; F - Final Sample Only; 5 - All 5 Samples; -- - No samples

Gas samples were analyzed for oxygen and carbon dioxide concentrations using an SRI GC equipped with a CTR-I concentric column (Altech) connected to a thermal conductivity detector (TCD). An isothermal method at ambient temperature was used with helium as the carrier gas. A 2-mL sample volume was injected through a multiport valve injector assembly. The concentrations of oxygen and carbon dioxide were calculated using response factors generated from a multipoint calibration from injections of standards of known concentration.

4.2.2 Petroleum Hydrocarbons in Gas Samples

Gas samples collected during atmosphere exchanging were analyzed for petroleum hydrocarbons using a HP 5890 GC equipped with a 60-m DB-1 widebore capillary column (J &W Scientific) connected to a flame ionization detector (FID). A six-port valve injection port equipped with a heated sorbent trap was used to introduce up to 2 mL of sample into the GC. The initial oven temperature was held at 20°C for 4 minutes, then ramped at 10°C/min up to 180°C and held for 12 minutes. The GC method was adjusted to account for sample-specific requirements to maintain acceptable detection limits. The data were collected on a HP 3392A integrator, and the concentrations of the specific hydrocarbons were calculated by multiplying the resulting area counts for each compound by the response factor. The response factor was calculated by

dividing a known concentration of each of the 20 compounds by the area count. The area count was determined by injecting a calibration standard at that concentration.

4.2.3 Petroleum Hydrocarbons in Soil Samples

Soil samples were analyzed on a HP 5890 GC using a heated purge-and-trap method. The GC was equipped with a 30-m DB-1 widebore capillary column connected to a FID. Approximately 1 g of soil was combined with 5 mL of blank water in the purge vessel. The initial oven temperature was held at 20°C with cryogen for 4 minutes, then ramped at 10°C/min to 240°C and held for 4 minutes or until a stable baseline was achieved. The peak elution and the resulting area counts were recorded on a HP 3396 Series II integrator. The concentrations of the 20 compounds listed in the calibration mixture were calculated by applying response factors determined from responses from injections of known concentration.

4.2.4 Soil Moisture

Moisture analysis was conducted on soil samples to determine whether soils contained suitable water content to support microbial growth and nutrient transfers. A gravimetric method (Gardner, 1965) was used to make this determination. Soil moisture analyses were conducted as follows:

Step 1.	Turn on the drying oven and allow the temperature to equilibrate at 105°C.
Step 2.	Preweigh and label drying dishes.
Step 3.	Weigh out a 5-g aliquot of soil in triplicate and place in the preweighed, labeled drying dish.
Step 4.	Place dishes containing the soil sample into the drying oven and allow them to dry for 24 hours.
Step 5.	Remove dishes and place them into a desiccator at room temperature and allow the sample dishes to cool to constant weight.
Step 6.	Remove the sample dishes from the desiccator and record the cumulative weight of the dish plus the sample after drying.

4.2.5 Soil pH

The pH of the soil from each experimental condition was measured before reactor setup, and the pH of soil from each reactor after incubation was determined using a method based on EPA SW-846 Method 9045A using a Cole-Parmer® Chemcadet® pH meter and glass electrode. The primary modification to SW-

846 Method 9045A was the reduction in the volume of material that was analyzed. The pH of the soils was determined as follows:

Step 1. Calibrate the pH meter using a dual-point calibration with pH 4.00 and pH 7.00 standards. Check the calibration slope to make sure the probe is in good working order.

Step 2. Add 10 g of soil to 10 mL of reagent water in a 50-mL beaker. Cover the beaker with parafilm, then mix the solution for 1 minute on a stirring plate using a Teflon™-coated magnetic stirring bar.

Step 3. Insert the electrode into the soil/water suspension and record the displayed pH value.

4.2.6 Dehydrogenase Activity in Soil Samples

Dehydrogenase activity was measured before inoculation and following the 60-day incubation period. The data were used to make a relative comparison of the microbial activity between experimental conditions. Dehydrogenase activity was measured according to the method described by Tabatabai (1982). The procedure was as follows:

Step 1. Prepare a 3% triphenyltetrazolium chloride (TTC) solution by dissolving 3 g of 2,3,5-TTC in 80 mL of water then adjusting the volume to 100 mL.

Step 2. Prepare a triphenyl formazan (TPF) standard solution by dissolving 100 mg of TPF in about 80 mL of water then bringing the volume up to 100 mL.

Step 3. Prepare a set of calibration standards by diluting 10 mL of the TPF standard solution with 100 mL with methanol. Pipette 5-, 10-, 15-, and 20-mL aliquots of this solution into 100-mL volumetric flasks and bring the volumes up to 100 mL with methanol to prepare standards of 500, 1,000, 1,500, and 2,000 µg of TPF/100 mL, respectively. Measure the intensity of the reddish color on a spectrophotometer at a wavelength of 485 nm. Generate a calibration curve by plotting the absorbance readings against the concentration of TPF.

Step 4. Thoroughly mix 10 g of soil and 0.1 g of $CaCO_3$, and place 3 g of this mixture into each of three 16 by 100 mm test tubes. Add 0.5 mL of the 3% TTC

solution and 1.25 mL of distilled water to each tube. Mix each tube with a glass rod, stopper the tubes, then incubate for 24 hr at 37°C.

Step 5. Following incubation, add 5 mL of methanol and shake for 1 minute, then filter through a glass funnel plugged with absorbent cotton into a 50-mL volumetric flask.

Step 6. Rinse the tube twice with 5 mL of methanol and add the rinsate to the filter.

Step 7. Rinse the funnel and cotton with 5-mL aliquots of methanol until the reddish color disappears, then dilute the filtrate to 50 mL volume with methanol.

Step 8. Measure the intensity of the reddish color using a spectrophotometer at a wavelength of 485 nm and determine the amount of TPF produced by comparison against the calibration curve prepared in Step 3.

4.2.7 Microbial Enumerations in Soil Samples

Microbial enumerations were conducted to determine how the microbial amendment either adds to or stimulates the growth of indigenous microorganisms. Heterotrophic enumerations were conducted in triplicate using a serial dilution and pour plate method based on Standard Method 9215B (Greenberg et al., 1992). The procedure for this analysis was conducted as follows:

Step 1. Prepare a basal inorganic medium by adding 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.05 g $CaSO_4 \cdot 2H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 1.0 g $(NH_4)_2SO_4$ into 1.0 L of distilled water and adjusting the pH to 7.2.

Step 2. Repeat Step 1 and add 20 g of a purified agar (noble agar) to the medium. Stir with heat until the agar has dissolved. Prepare a series of test tubes containing this medium by transferring a 20-mL aliquot to each 30-mL borosilicate glass test tube. Sterilize the medium by autoclaving. After sterilization, store the medium in a 45°C water bath.

Step 3. Prepare a series of dilution blanks by dispensing 9 mL of the basal inorganic medium prepared in Step 1 into 20-mL borosilicate glass test tubes. Prepare a separate set of dilution blanks by dispensing 10 mL of the basal inorganic medium prepared in Step 1 into 20-mL borosilicate glass test tubes. Sterilize both sets of dilution blanks in an autoclave. Store the dilution tubes in a

refrigerator at 10°C until they are ready to be used. Allow the tubes to equilibrate to room temperature before using.

Step 4. Label the outside of each sterile petri plate with the appropriate sample, dilution, and replicate information.

Step 5. Weigh out, in triplicate, a 1-g aliquot of soil, and transfer it to a dilution blank containing 10 mL of phosphate buffer solution, and vortex for 30 seconds. Aseptically transfer 1 mL of this suspension to a dilution blank containing 9 mL of buffer and vortex the suspension for 5 seconds. Continue with this dilution process until the desired number of dilutions have been made.

Step 6. Aseptically transfer 1 mL of each dilution into the bottom portion of the appropriate petri plate.

Step 7. Aseptically add a 10-µL aliquot of JP-4 jet fuel into each tube containing 20 mL of mineral salts/noble agar medium using a 25-µL gastight syringe, and mix by gently swirling each tube so that bubbles are not formed. Aseptically transfer the contents of each tube into the bottom portion of each inoculated petri plate. Swirl the contents of each plate thoroughly to mix the sample while taking care not to splash the mixture over the edge. Allow the contents to solidify.

Step 8. After solidification invert each petri dish and transfer into a plastic bag. Add 10 µL of JP-4 jet fuel onto a paper towel and lay the paper towel on the top of the plates and seal the plastic bag with tape.

Step 9. Incubate plates at 25 °C until countable growth is apparent (approximately 5 to 7 days).

Step 10. After the incubation period, observe plates for bacterial growth.

4.2.8 Nutrient Concentrations in Soil Samples

Soils were sent to A&L Analytical Laboratories, Inc. in Memphis, Tennessee for nutrient analysis. Ca, Mg, K, and Na were extracted with excess NH_4OAc and analyzed for by atomic absorption (AA) spectroscopy. Phosphorous was measured using the Fiske-Subbarrow version of the Bray-1 extraction method. Phosphorous was extracted using hydrochloric acid and ammonium fluoride, then color development was measured using a Gilford spectrophotometer at 660 nm. Total Kjeldahl nitrogen was measured by digesting soil samples for 5 hours in concentrated sulfuric acid using a catalyst and distilling the samples in

removal of excess ammonium present in the soil as the acetate, the exchangeable ammonium was determined by displacement with NaCl and distillation. A detailed description of the method for cation exchange capacity analysis is contained in Appendix A.

4.3 Results

The analytical protocols conducted during Experiment #1, as described in Section 4.2, were completed and the data were reduced and evaluated. The results from each protocol are presented in the following sections along with a discussion of the trends between experimental conditions, the added value of the data obtained using the specified protocols, and any method modifications required to enhance the data obtained.

4.3.1 Oxygen and Carbon Dioxide in Gas Samples

The cumulative oxygen consumption of the uncontaminated soil is exhibited in Figure 3. All reactors showed a steady increase of oxygen consumption over time. Reactor Control 1 A cumulatively consumed the most oxygen out of the three reactors with uncontaminated soil. The final cumulative oxygen consumed after 60 days was 30.53 mg. Reactor Control 1 A had the second lowest amount of carbon dioxide produced. No carbon dioxide was produced the first day, but after 5 days the carbon dioxide produced was 0.94 mg. The cumulative carbon dioxide produced leveled off on day 12 at 2.55 mg.

Reactor Control 1 B showed a decrease of consumed oxygen at day 35. At the sampling on day 35, Control 1 B had a reading of 22.40% oxygen or 2.00 mg of oxygen produced. After day 35, the cumulative oxygen consumption increased again. On day 49, the 4.50 mg of oxygen consumed made the cumulative oxygen consumption of Control 1 B comparable to the oxygen consumption levels of Control 1 A and Control 1 C. Control 1 B had the greatest cumulative amount of carbon dioxide produced among the reactors with uncontaminated soil. The cumulative carbon dioxide leveled off after 12 days at 2.79 mg. After 32 days, the cumulative carbon dioxide produced increased to 5.79 mg. The cumulative carbon dioxide produced for Control 1 B leveled off at 5.93 mg after 42 days.

The cumulative oxygen consumption of Reactor Control 1 C was the lowest until day 35, when Control 1 B dropped. The final cumulative oxygen consumption was 29.07 mg. The greatest jump was from day 49 to day 54, when 8.13 mg of oxygen was consumed in that time frame. The carbon dioxide produced was the lowest of the three reactors with uncontaminated soil. No carbon dioxide was produced the first day.

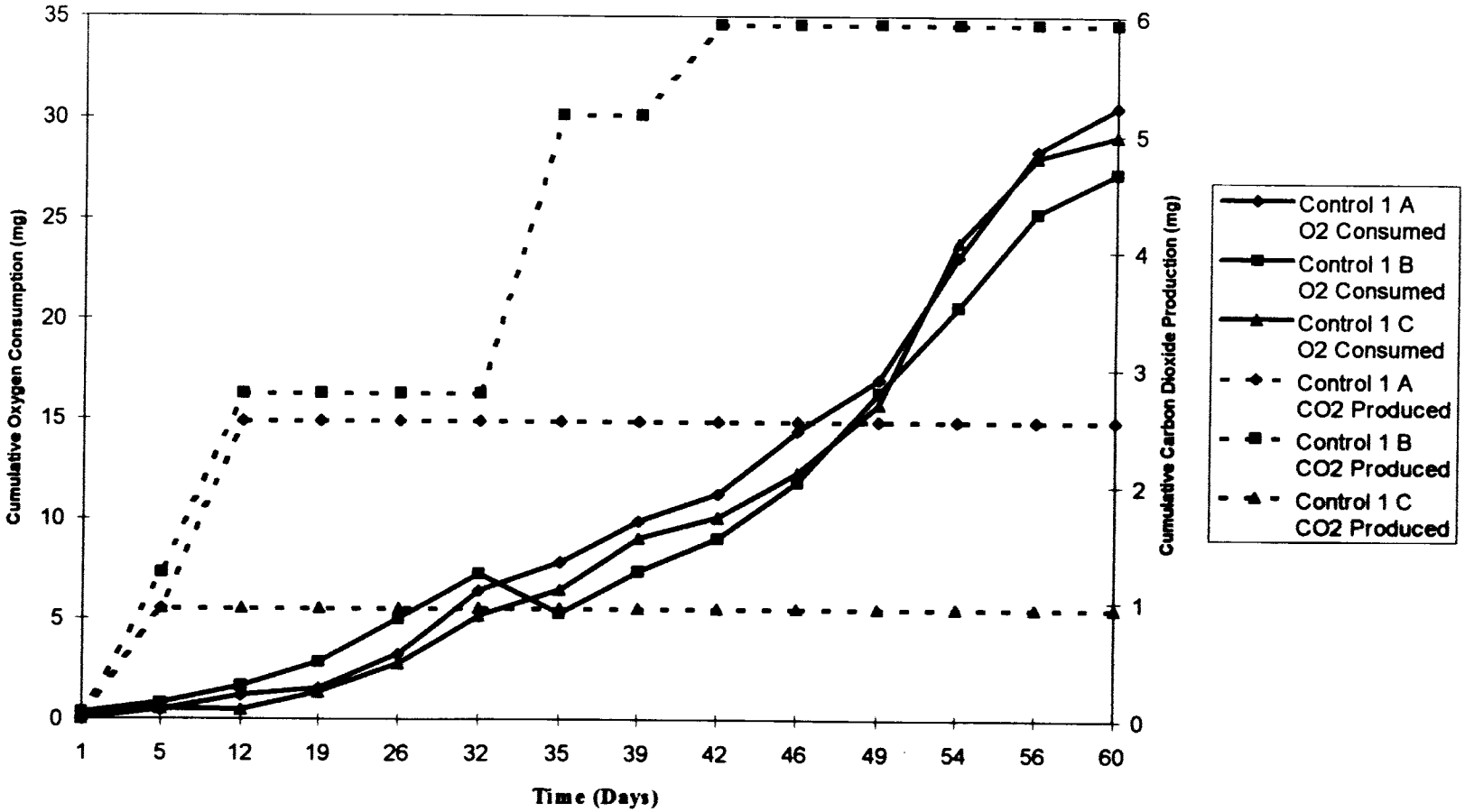

Figure 3. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production in Reactors Containing Uncontaminated Soil During Experiment #1.

On the fifth day, 0.94 mg of carbon dioxide was produced, but no more carbon dioxide was detected after day 5. The GC used to monitor the carbon dioxide and oxygen needs to be more sensitive to detect the lower levels of these compounds, or the calibration gas used needs to contain the lower levels of carbon dioxide and oxygen that are seen in the samples.

The cumulative consumption of oxygen and cumulative production of carbon dioxide for contaminated soil with sterilized amendment (Control 2) is shown in Figure 4. Control 2 A was lost when the column broke. The contaminated soil with sterilized amendment had higher cumulative amounts of oxygen consumption and carbon dioxide production than the uncontaminated soil. The average total oxygen consumption of the uncontaminated soil was 28.95 mg after 60 days, while the average total oxygen consumption of the contaminated soil with the sterilized amendment was 56.47 mg in the same time. The average cumulative consumption of oxygen for the five conditions in Experiment #1 is exhibited in Figure 8. The average total carbon dioxide production of the uncontaminated soil was 3.14 mg and the average total carbon dioxide production of the contaminated soil with sterilized amendment was 32.44 mg, a 10% difference.

Control 2 B had a higher cumulative consumption of oxygen and production of carbon dioxide than Control 2 C. Control 2 B showed a steady increase of oxygen consumption over time. The cumulative consumption of oxygen after 60 days was 66.01 mg. The cumulative carbon dioxide produced was 41.96 mg. Control 2 C had a cumulative oxygen consumption of 43.39 mg and a cumulative carbon dioxide production of 22.92 mg. Control 2 B produced twice as much carbon dioxide as Control 2 C.

The cumulative oxygen consumption and cumulative carbon dioxide production in reactors with contaminated soil without any amendment is shown in Figure 5. The average cumulative oxygen consumed was greater under this condition than for the contaminated soil with the sterilized amendment. The average total oxygen consumed when the sterilized amendment was added was 56.47 mg and the average total oxygen consumed for the contaminated soil without any amendment was 66.69 mg. The average total carbon dioxide produced for the contaminated soil with the sterilized amendment and that produced for the contaminated soil without amendment were 32.44 mg and 34.07 mg, respectively.

Reactor 1 A had the lowest cumulative amount of oxygen consumed among the reactors containing only contaminated soil. On day 11, there was 21.36% oxygen or 1.08 mg of oxygen produced. This created a negative quantity of cumulative oxygen consumed. On day 26, the 8.83 mg of oxygen consumed made the cumulative oxygen consumed for Reactor 1 A comparable to that consumed for Reactor 1 B and Reactor 1 C. Reactor 1 A also had the lowest carbon dioxide produced, which is to be expected if the oxygen consumed was the least. The total carbon dioxide produced was 3,3.39 mg.

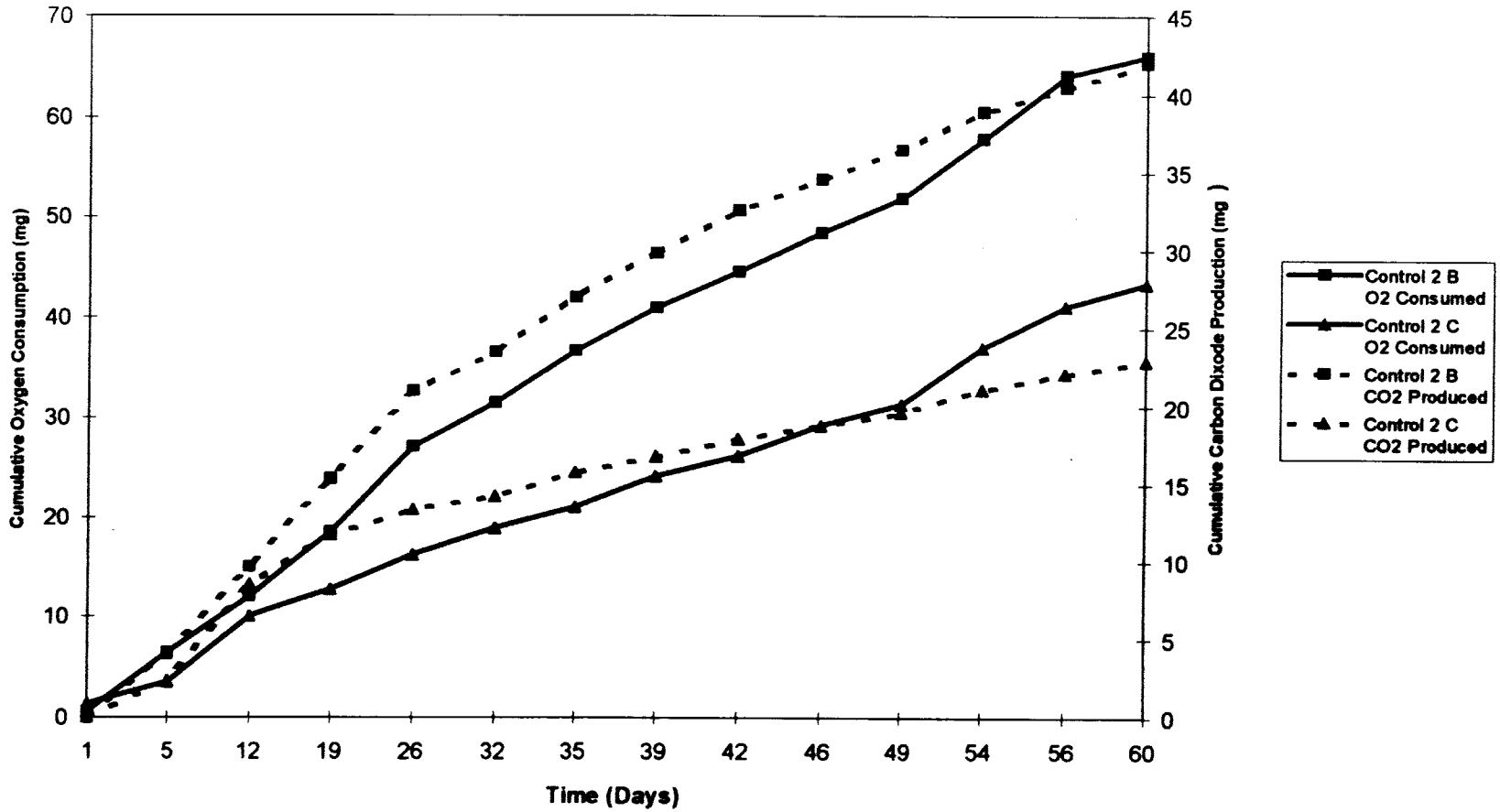

Figure 4. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production in Reactors Containing Contaminated Soil and Sterilized Amendment During Experiment #1.

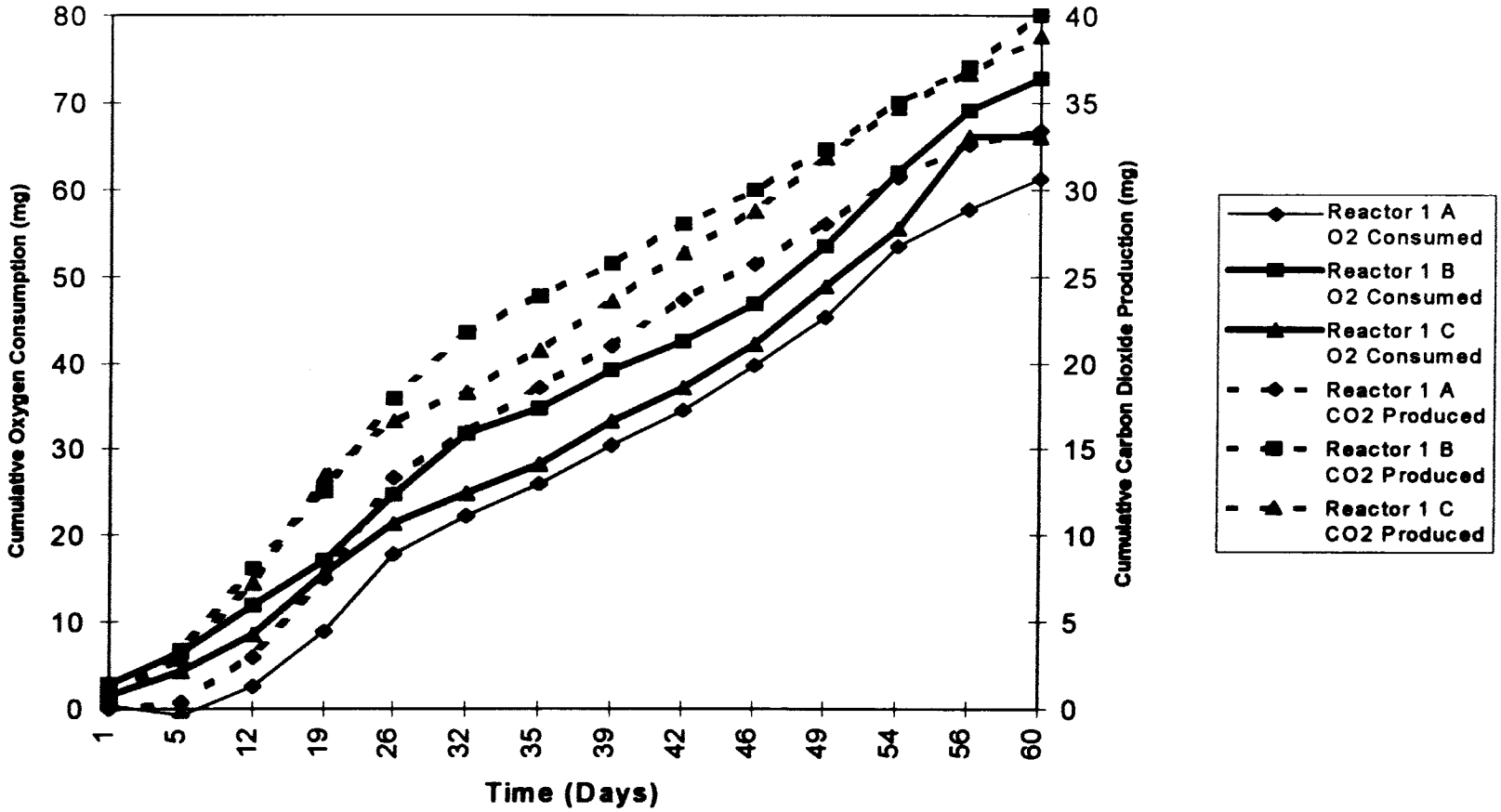

Figure 5. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production in Reactors Containing Contaminated Soil During Experiment #1.

Reactor 1 B had the highest consumption of oxygen and production of carbon dioxide throughout the 60 days of the experiment. The cumulative oxygen consumption increased throughout the experiment and the total consumption of oxygen was 72.83 mg. The production of carbon dioxide increased throughout the 60 days of the experiment and the total production of carbon dioxide was 39.99 mg. Reactor 1 C had a total of 66.07 mg of oxygen consumed and 38.83 mg of carbon dioxide produced. Both the carbon dioxide production and the oxygen consumption increased over time.

The data for the cumulative consumption of oxygen and the cumulative production of carbon dioxide for the uncontaminated soil with microbial amendment and JP-4 addition are displayed in Figure 6. Reactor 2 A had the highest consumption of oxygen at 80.23 mg. This reactor was approximately equal with the other reactors until day 39 when oxygen consumption exceeded that of the other two reactors. Although oxygen consumption for the other reactors continued to increase also, that of Reactor 2 A increased a greater amount over the last 21 days. The carbon dioxide production of Reactor 2 A showed the same trend as the cumulative oxygen consumption. The total carbon dioxide produced was 48.13 mg.

Reactor 2 B had a total oxygen consumption of 73.44 mg. Reactor 2 C had the lowest oxygen consumption at a total of 68.33 mg. Reactor 2 B and Reactor 2 C had equal amounts of carbon dioxide produced, 42.86 mg and 42.96 mg, respectively.

The cumulative oxygen consumption and carbon dioxide production data from the contaminated soil with the microbial amendment are exhibited in Figure 7. The cumulative oxygen consumption data for the three reactors were close throughout the experiment. The data points for the production of carbon dioxide for all three reactors were close until day 32, when the production of carbon dioxide in Reactor 3 A fell behind that of Reactor 3 B and Reactor 3 C. On day 42, the Reactor 3 B production of carbon dioxide jumped ahead of that for Reactor 3 C and the tightness of the carbon dioxide production data ceased to exist, although both reactors had the same increasing trend.

The cumulative consumption of oxygen and production of carbon dioxide for Reactor 3 A increased throughout the 60 days of the experiment. Reactor 3 A had the lowest oxygen consumed among the three reactors. The total oxygen consumed was 70.59 mg. Reactor 3 A also had the lowest amount of carbon dioxide produced, 38.83 mg.

Reactor 3 B had a total of 74.26 mg of oxygen consumed and a total of 51.68 mg of carbon dioxide produced. The carbon dioxide produced was the largest of the three reactors with contaminated soil and the microbial amendment addition. Reactor 3 C had a total of 76.43 mg of oxygen consumed, the highest of the three reactors. The total carbon dioxide produced from Reactor 3 C was 43.41 mg.

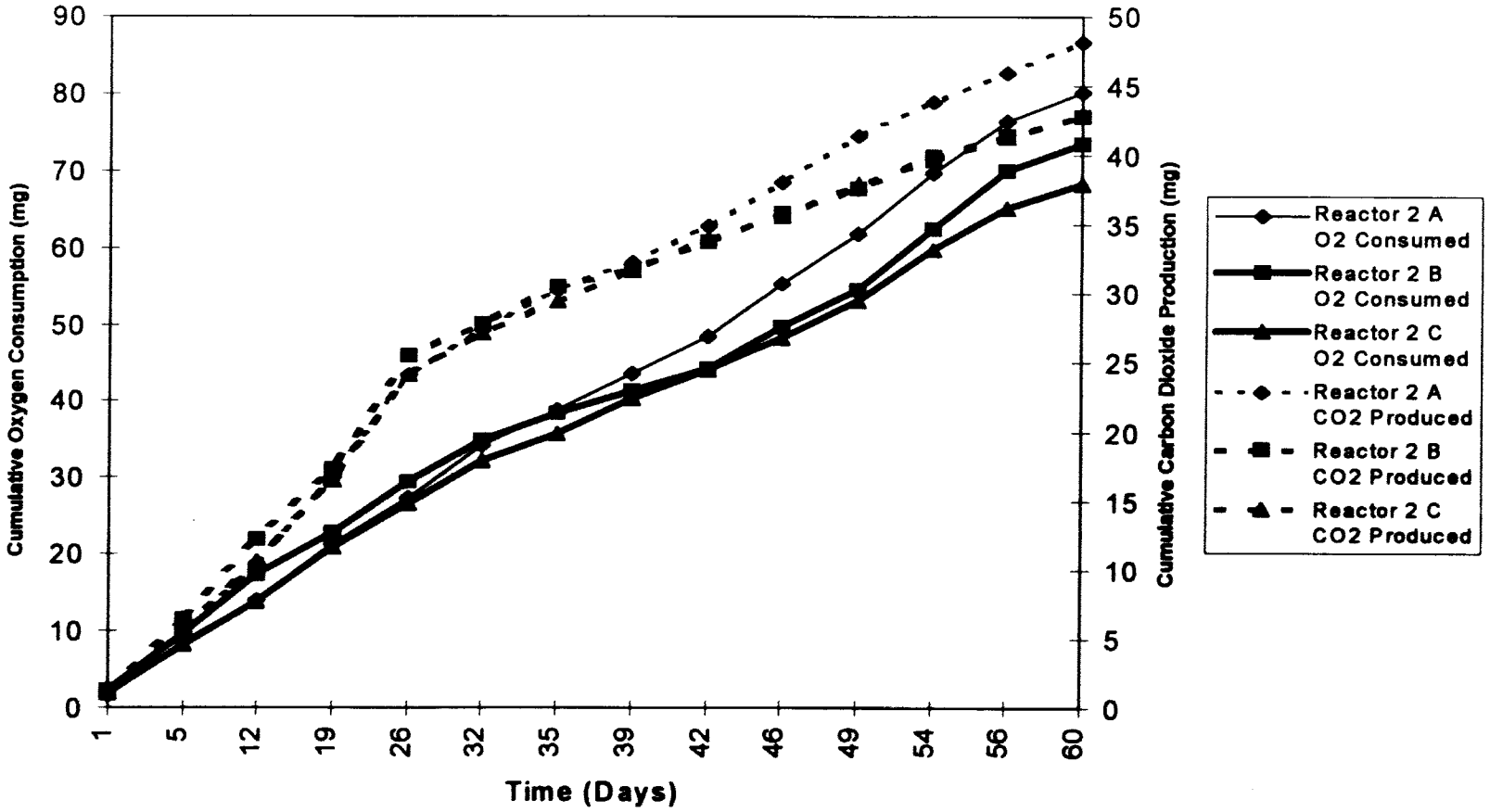

Figure 6. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production in Reactors Containing Uncontaminated Soil with Amendment and JP-4 Addition During Experiment #1.

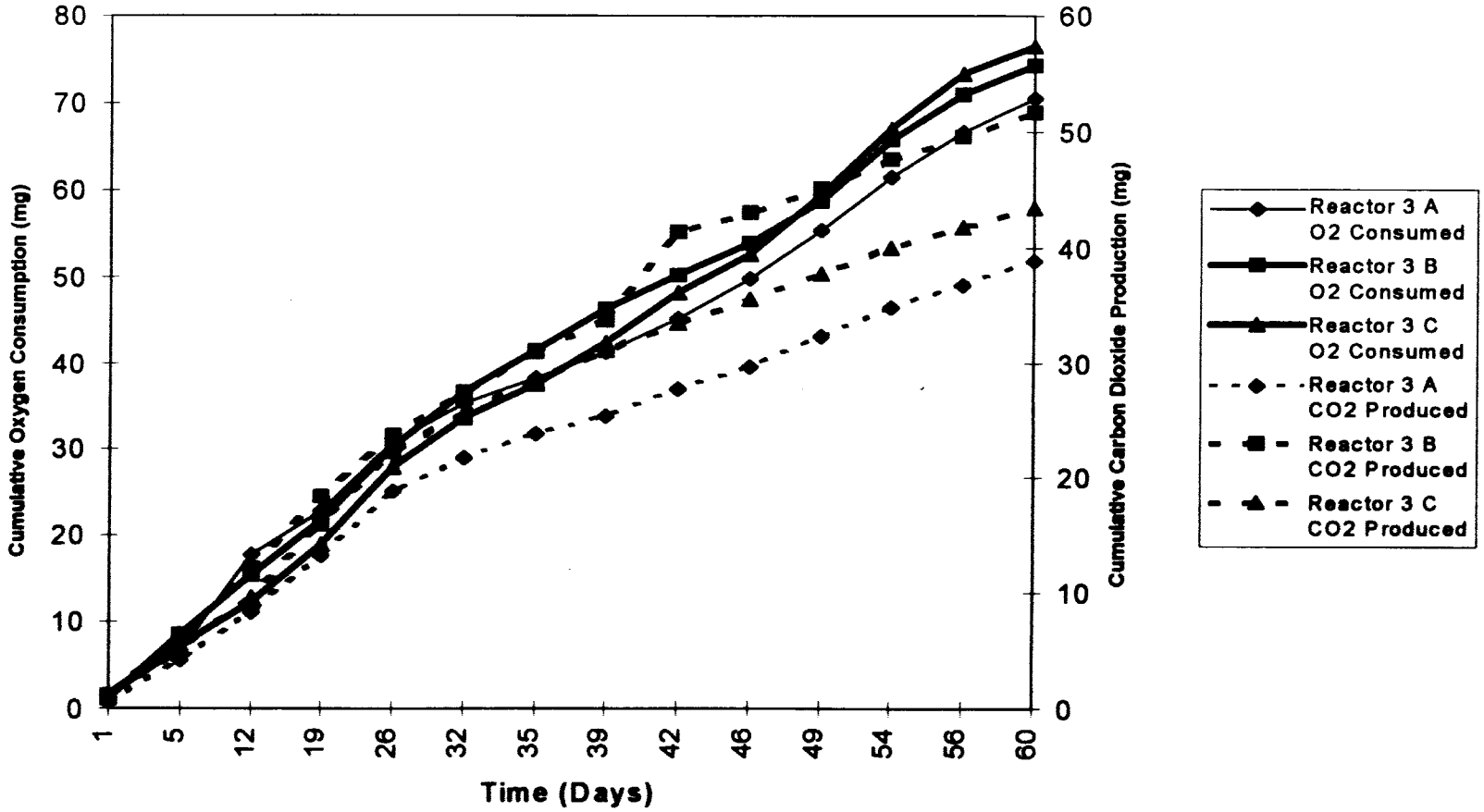

Figure 7. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production in Reactors Containing Contaminated Soil with Amendment During Experiment #1.

Figure 8 shows the average cumulative consumption of oxygen for all conditions of the first experiment. Control 1 (uncontaminated soil) had a total of 28.95 mg of oxygen consumed, the lowest average total of oxygen consumed among the five conditions. The uncontaminated soil had the lowest microbial activity. The microbial population did not grow, but stayed essentially the same (Section 4.2.7, Table 9).

Control 2 had the second lowest total of oxygen consumed, with a value of 56.47 mg. Control 2 was the contaminated soil with sterilized amendment. Here, the indigenous microbial population was responsible for any degradation of the petroleum hydrocarbons present in the soil.

Reactor 1 had average oxygen consumption levels comparable to Control 2 until day 49. The oxygen consumption then greatly increased in Reactor 1, giving an average total of 66.69 mg of oxygen consumed. It was interesting that the oxygen consumed in Reactor 1 containing only the contaminated soil was higher than in the reactors receiving the sterilized microbial amendment that contained the nutrients from the amendment product.

The total consumption of oxygen in Reactor 2 was nearly equal to that in Reactor 3, the totals being 74.00 mg and 73.76 mg, respectively. Reactor 2 is the uncontaminated soil with amendment and JP-4 addition and Reactor 3 is the contaminated soil with amendment. The data of the average consumption of oxygen between these two reactors were equal all the way through the experiment. Reactor 2 and Reactor 3 had the highest amount of oxygen consumed. The average amounts of carbon dioxide produced for Reactor 2 and Reactor 3 were equal at 44.65 mg and were higher than for the other three conditions.

4.3.2 Petroleum Hydrocarbons in Gas Samples

Figure 9 shows the cumulative total petroleum hydrocarbons (TPH) in gas samples from Control 1, the uncontaminated soil. The three reactors with uncontaminated soil began with the same amount of TPH and the TPH increased over time. Sampling on day 19 showed an increase for Control 1 A and Control 1 C. Control 1 A jumped up to 7.30 μg and Control 1 C to 6.80 μg, while Control 1 B went to 2.14 μg cumulative TPH. The cumulative TPH for Control 1 A increased on day 26 to 12.50 μg. During the rest of the sampling, the cumulative TPH for Control 1 A had no more large increases, but leveled off at 15.70 μg on day 54 and stayed there for the remaining 6 days. Control 1 A had the largest cumulative TPH value among the three reactors with uncontaminated soil.

The cumulative TPH data for gas samples from contaminated soil with the sterilized amendment are shown in Figure 10. Control 2 A was lost when the column broke. Control 2 B and Control 2 C showed the same trends. From day 1 to day 19, both increased their TPH by almost seven times the initial TPH. From

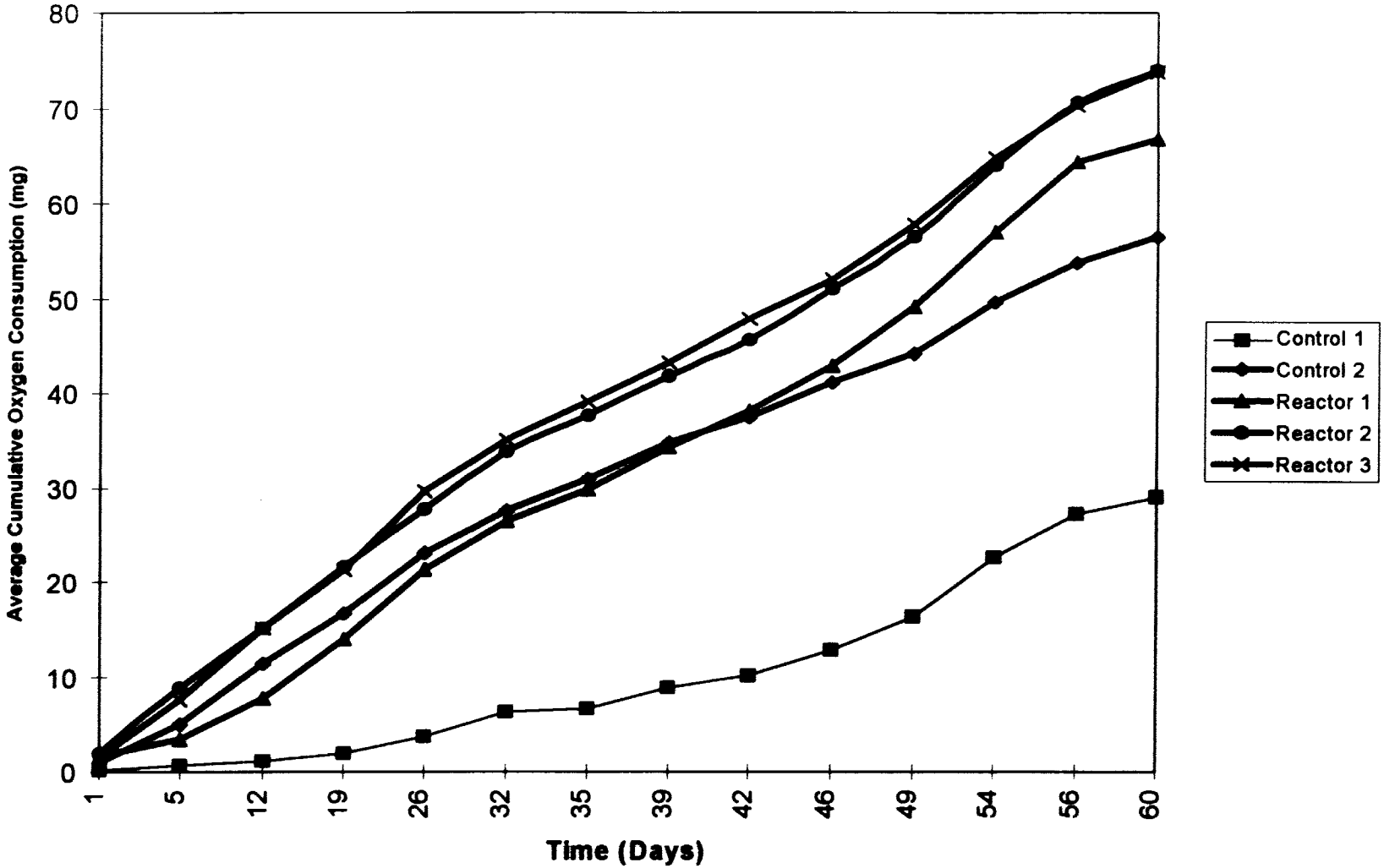

Figure 8. Average Cumulative Oxygen Utilization in Reactors Containing Soil Under the Five Conditions During Experiment #1.

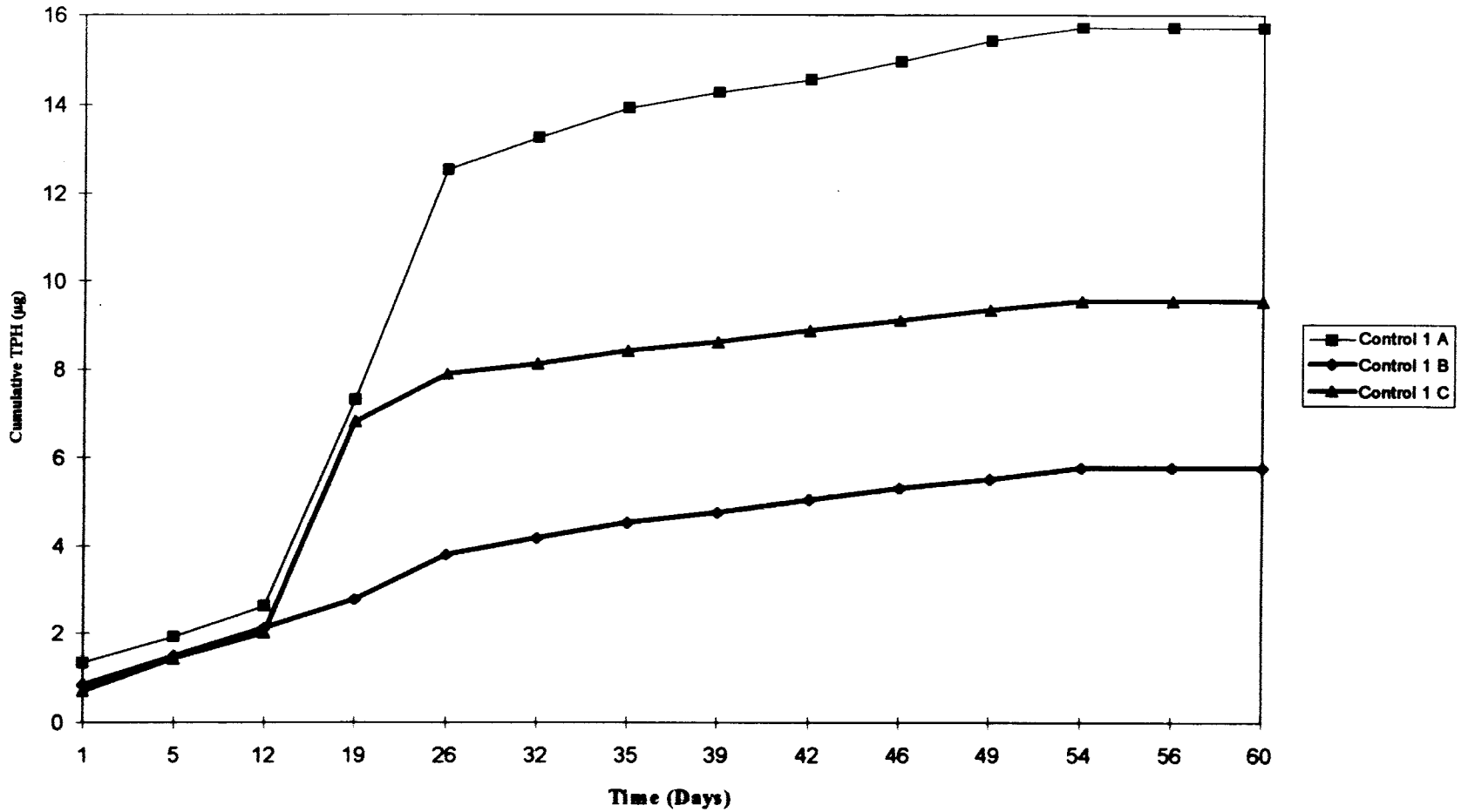

Figure 9. Cumulative Total Petroleum Hydrocarbons (TPH) Removed from Reactors Containing Uncontaminated Soil During Atmosphere Exchanging in Experiment #1.

day 26 to day 60, Control 2 B and Control 2 C leveled off around 15 µg to 17 µg. Control 2 B had a cumulative TPH of 15.88 µg and Control 2 C had a cumulative TPH of 16.92 µg. Similar to the Control 1 reactors, the cumulative TPH of the Control 2 reactors did not change after day 54, staying the same for 6 more days until the end of the experiment.

The data from the cumulative TPH of the contaminated soil without any amendment are exhibited in Figure 11. As with the Control 1 and Control 2 columns, the Reactor 1 columns reached their cumulative TPH value after 54 days and did not add any more TPH for the remaining 6 days of the experiment. Also, the trend of rapid TPH addition from day 1 to day 19 was seen in the gas sample data from the three reactors containing only the contaminated soil. Reactor 1 A had the lowest amount of cumulative TPH removed throughout this experiment. From day 1 to day 26, the cumulative TPH value increased by increments of 2 µg. After day 26, the TPH leveled out and increased by less than 1 µg each sampling. The final cumulative TPH for Reactor 1 A was 10.40 µg.

Reactor 1 B had the greatest cumulative TPH of the three reactors with only the contaminated soil. The total TPH was 19.52 µg. From day 1 to day 19, approximately 4 µg was added during each sampling day. After day26, less than 1 µg of TPH was added to the cumulative TPH for Reactor 1 B. Reactor 1 C had a cumulative TPH value of 14.33 µg.

The cumulative TPH data for the uncontaminated soil with microbial amendment and JP-4 addition are shown in Figure 12. Reactor 2 samples contained the highest levels of TPH among the five conditions tested in the first experiment because the JP-4 that was added was "fresh." Reactor 2 A had the highest cumulative TPH value at 3324.91 µg. Reactor 2 B had 3258.47 µg, and Reactor 2 C had 3019.63 µg of cumulative TPH. The same trend as the previous conditions was seen in the Reactor 2 columns. There was a steep increase in cumulative TPH values from day 1 to day 26. After day 26, the levels evened out. After day 54, no more TPH was added to the cumulative TPH. The three columns with the uncontaminated soil with microbial amendment and JP-4 addition had tight data points throughout the experiment.

Figure 13 shows the data for the contaminated soil with the microbial amendment addition. The levels of TPH began higher for this condition than for the Control 1, Control 2, and Reactor 1 conditions. Reactor 3 A had the lowest TPH values during the entire experiment. From the first to fifth, 2.35 µg was added to the cumulative TPH. On day 12 and day 19, approximately 2.50 µg was added. After day 26, less than 1 µg was added at each sampling time. The final TPH value was 24.01 µg, which was reached on day 56.

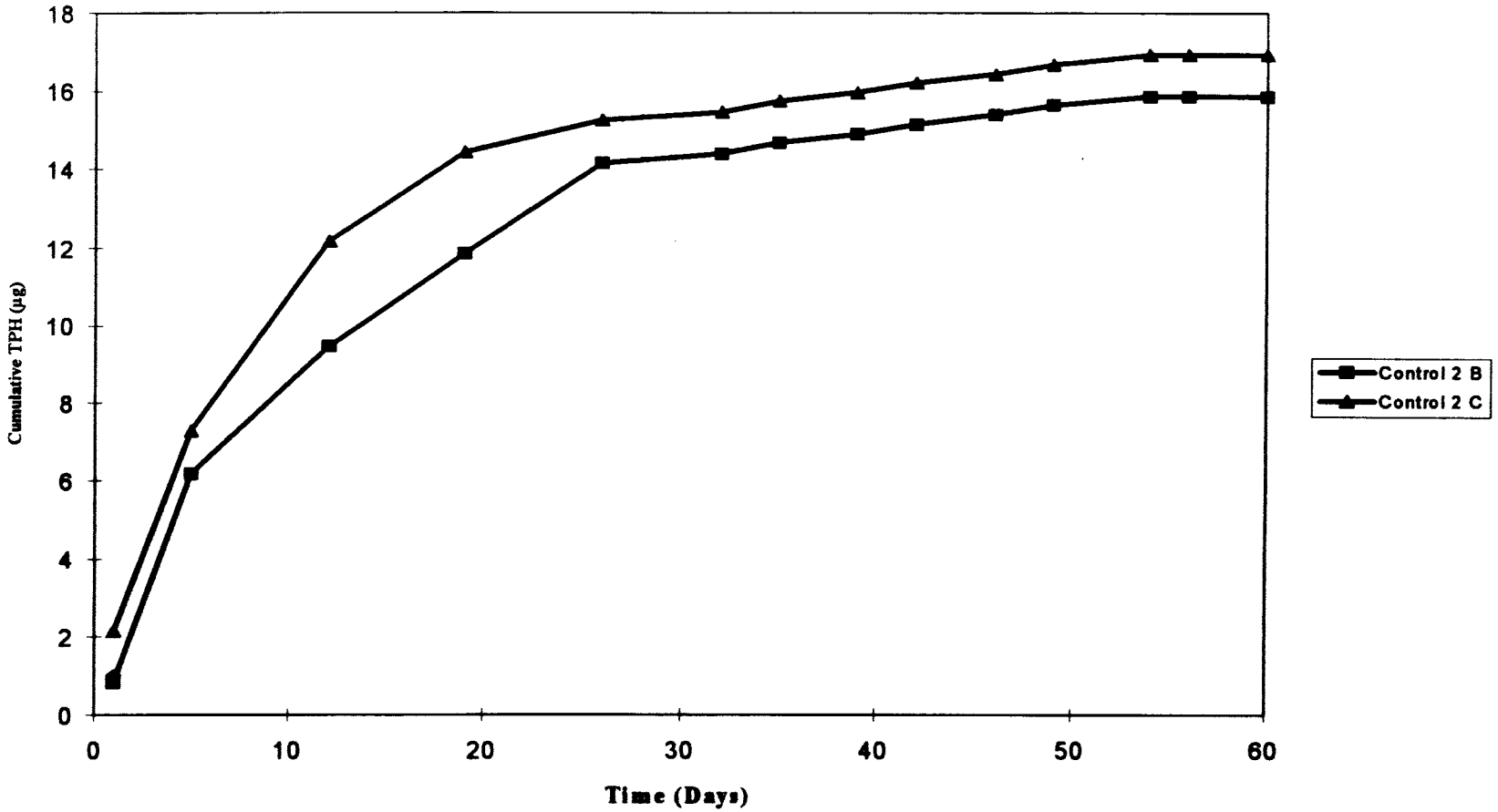

Figure 10. Cumulative TPH Removed from Reactors Containing Contaminated Soil and Sterilized Amendment During Atmosphere Exchanging in Experiment #1.

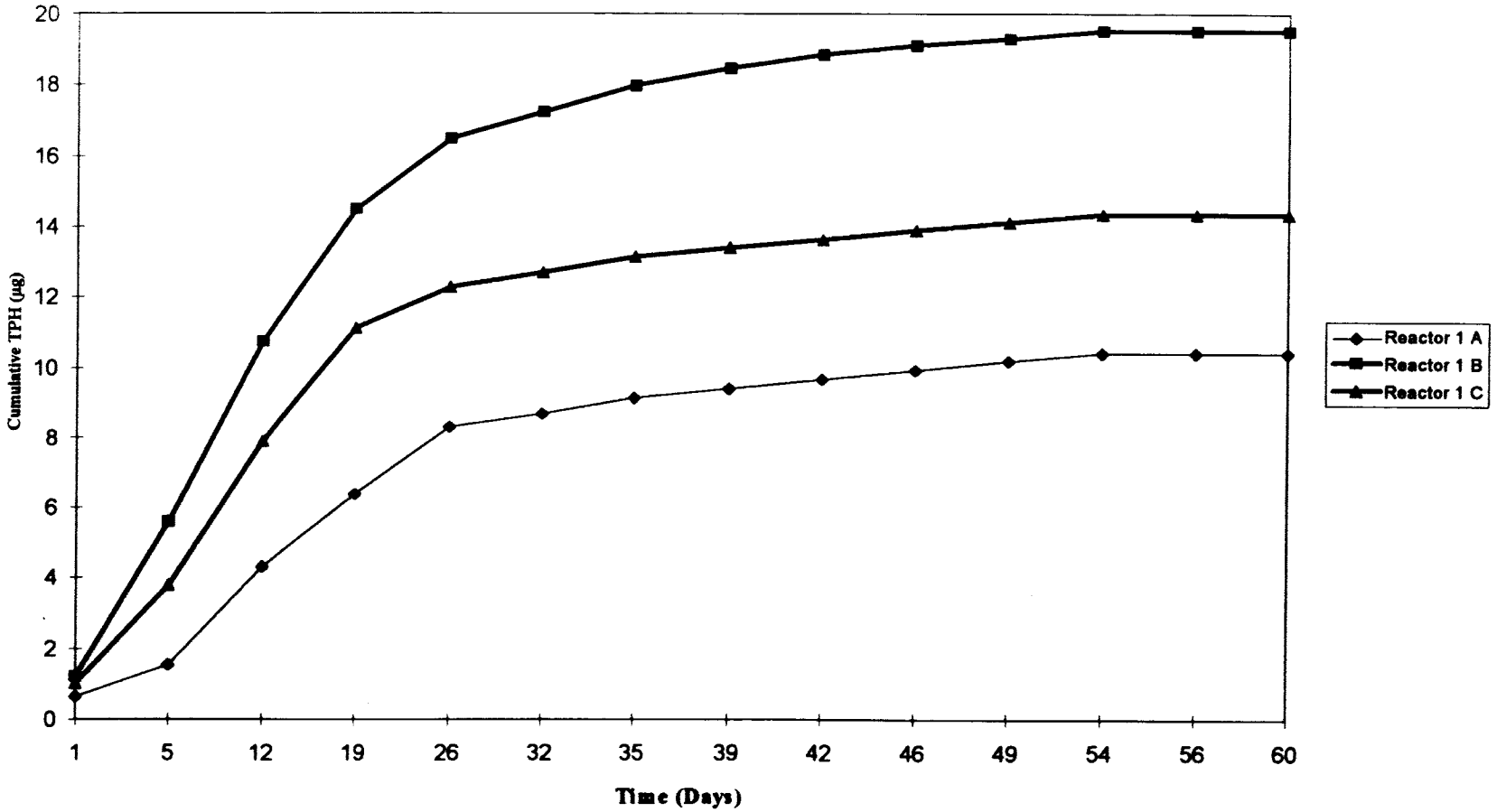

Figure 11. Cumulative TPH (µg) Removed from Reactors Containing Contaminated Soil During Atmosphere Exchanging in Experiment #1.

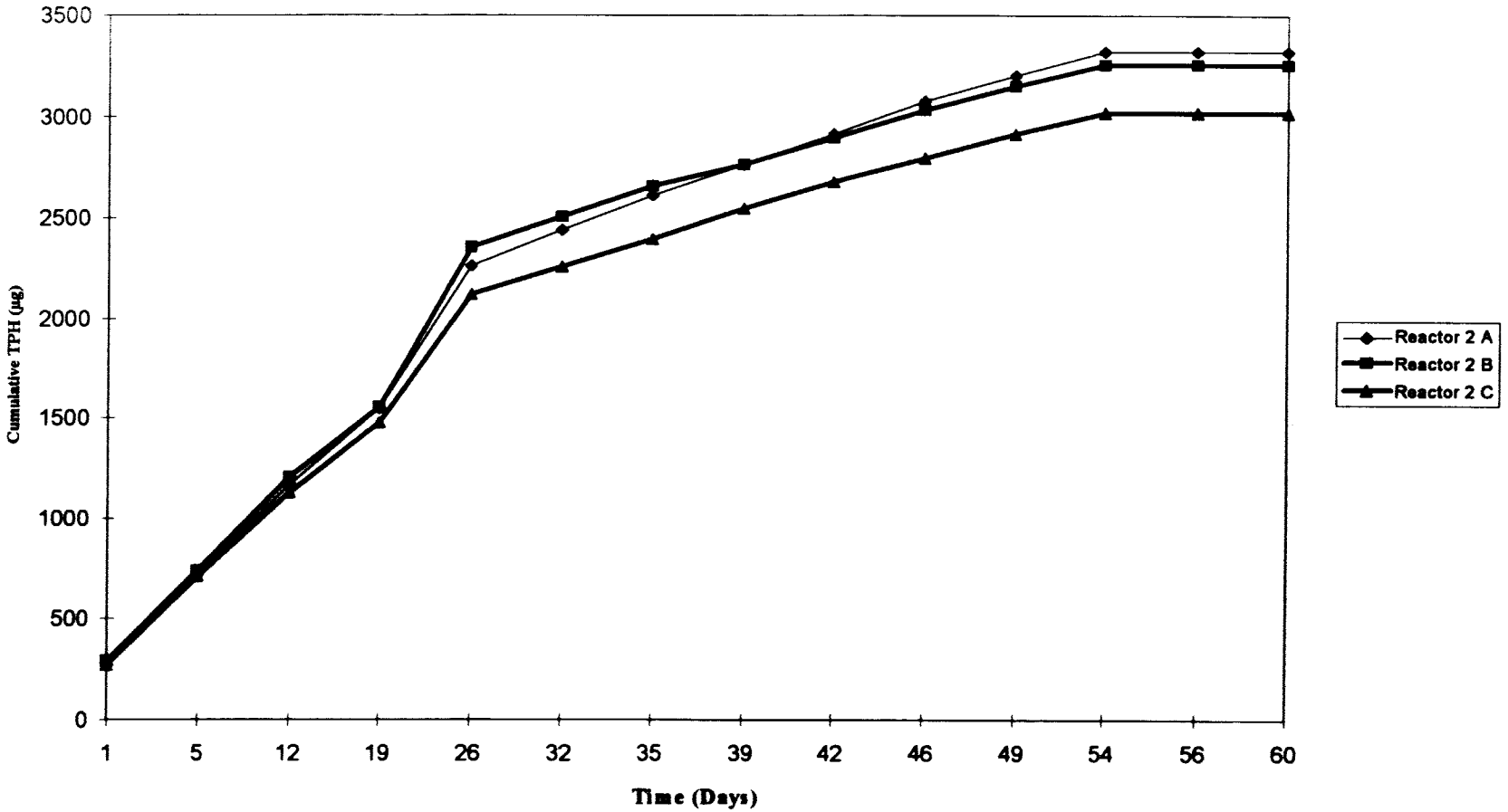

Figure 12. Cumulative TPH (µg) Removed from Reactors Containing Uncontaminated Soil with Microbial Amendment and JP-4 Addition During Atmosphere Exchanging in Experiment #1.

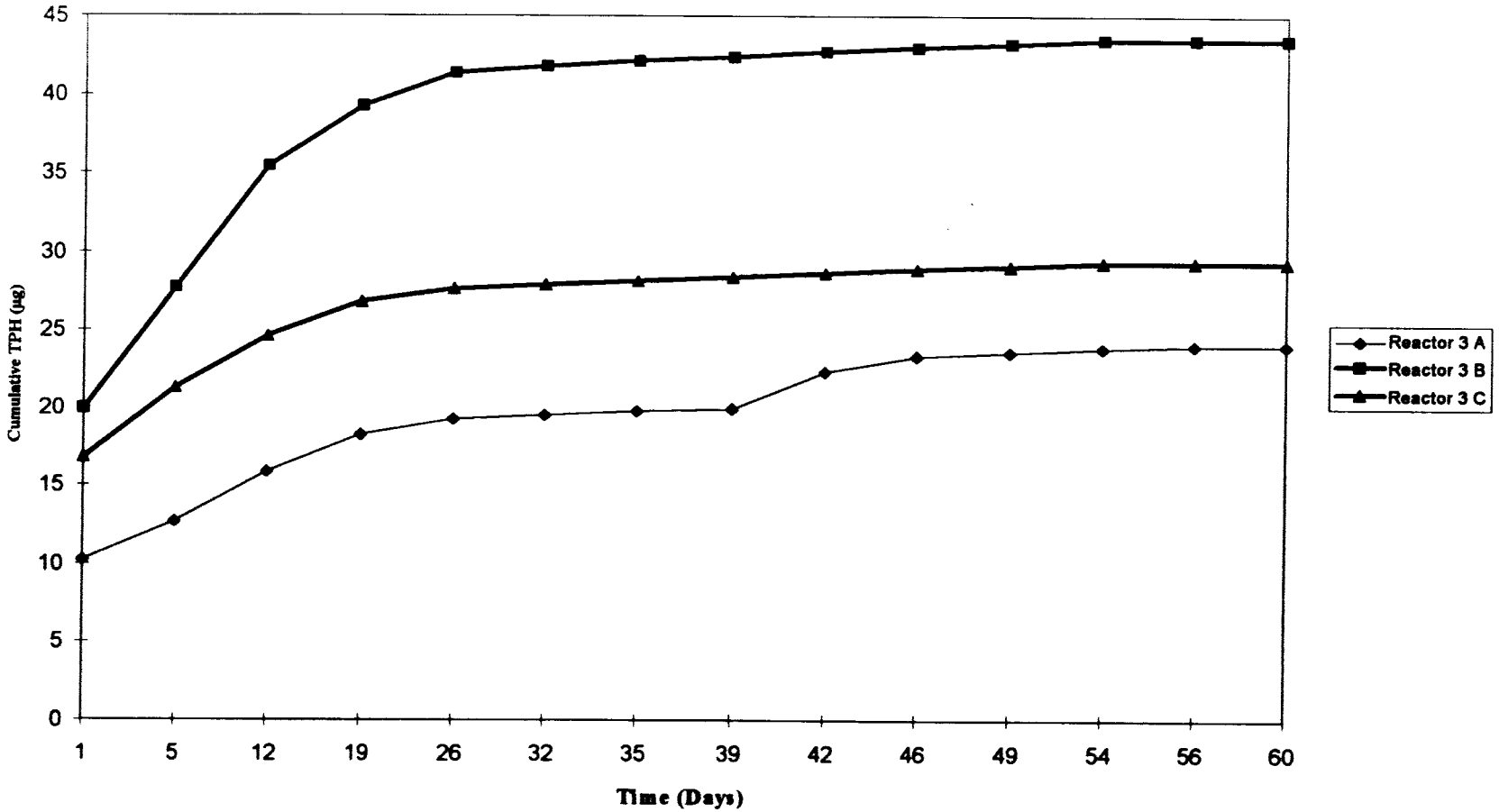

Figure 13. Cumulative TPH (µg) Removed from Reactors Containing Contaminated Soil with Microbial Amendment During Atmosphere Exchanging in Experiment #1.

Reactor 3 B had the highest cumulative TPH among the three columns with contaminated soil with amendment addition. The cumulative TPH value was 43.51 µg. The Reactor 3 C TPH began at 19.93 µg, higher than the TPH for the two other columns. After day 19, the TPH leveled out in the low 40s. The total TPH was reached on day 54. No TPH was added the remaining 6 days. The Reactor 3 C TPH leveled out after day 19 in the high 20s. The final TPH for Reactor 3 C was 43.51, which was reached on day 54.

4.3.3 Petroleum Hydrocarbons in Soil Samples

Table 4 shows the TPH content from the various conditions in Experiment #1. A negative average change indicates a final mass that is smaller than the initial TPH mass. The greatest change (–97.09%) took place in the contaminated soil with sterilized microbial amendment. The uncontaminated soil plus amendment and JP-4 had the highest initial mass of petroleum hydrocarbons but changed the least out of all of the conditions.

The bar graphs shown in Figures 14 through 18 show the mass of each petroleum hydrocarbon compound in each experimental condition. Figure 14 depicts the individual mass of petroleum hydrocarbon compounds in Control 1, the uncontaminated soil. The only compound detected in Control 1 was a minute amount (0.043 mg) of *n*-pentadecane, which was degraded completely. Figure 15 represents the levels of contamination in Control 2, which is the contaminated soil with sterilized amendment. The compounds trimethylbenzene, *n*-decane, *n*-butylbenzene, *n*-dodecane, *n*-tridecane, and *n*-tetradecane were reduced in mass from the initial values. The other compounds that were present at the beginning of the experiment were totally degraded by the end of the experiment.

The amounts of petroleum hydrocarbon compounds in the soil samples from Reactor 1 are displayed in Figure 16. The mass of toluene remained the same from the initial analysis to the final analysis. The *o*-xylene, ethylbenzene, trimethylbenzene, *n*-butylbenzene, and *n*-decane were totally degraded. The compound *n*-pentadecane was not detected in the initial analysis but was detected in the final analysis. The *n*-dodecane and *n*-tridecane masses decreased, but did not totally degrade. The compound *n*-tetradecane decreased slightly. The mass of petroleum hydrocarbons in Reactor 2 was four times greater than in any of the other experimental conditions (Figure 17). Furthermore, the quantity of petroleum hydrocarbon compounds observed in this reactor was greater. The masses of ethylbenzene, *n*-tridecane, *n*-tetradecane, and *n*-pentadecane were higher after the experimental run. The other components that were present at the start of the experiment were reduced after the 60-day experimental processing.

Table 4. Results of TPH Analysis Conducted on Soil Samples During Experiment #1.

Uncontaminated Soil with No Amendment				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
0.015	0.003	0.004	0.002	-80.00
Contaminated Soil with No Amendment				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
6.88	0.82	0.58	0.32	-91.67
Contaminated Soil with Sterilized Amendment Addition				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
7.21	NA	0.27	0.15	-97.09
Contaminated Soil with Amendment Addition				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
7.44	0.19	0.29	2.59	-86.25
Uncontaminated Soil with Amendment and JP-4 Addition				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Ave 40.90	13.44	11.21	19.73	-63.83

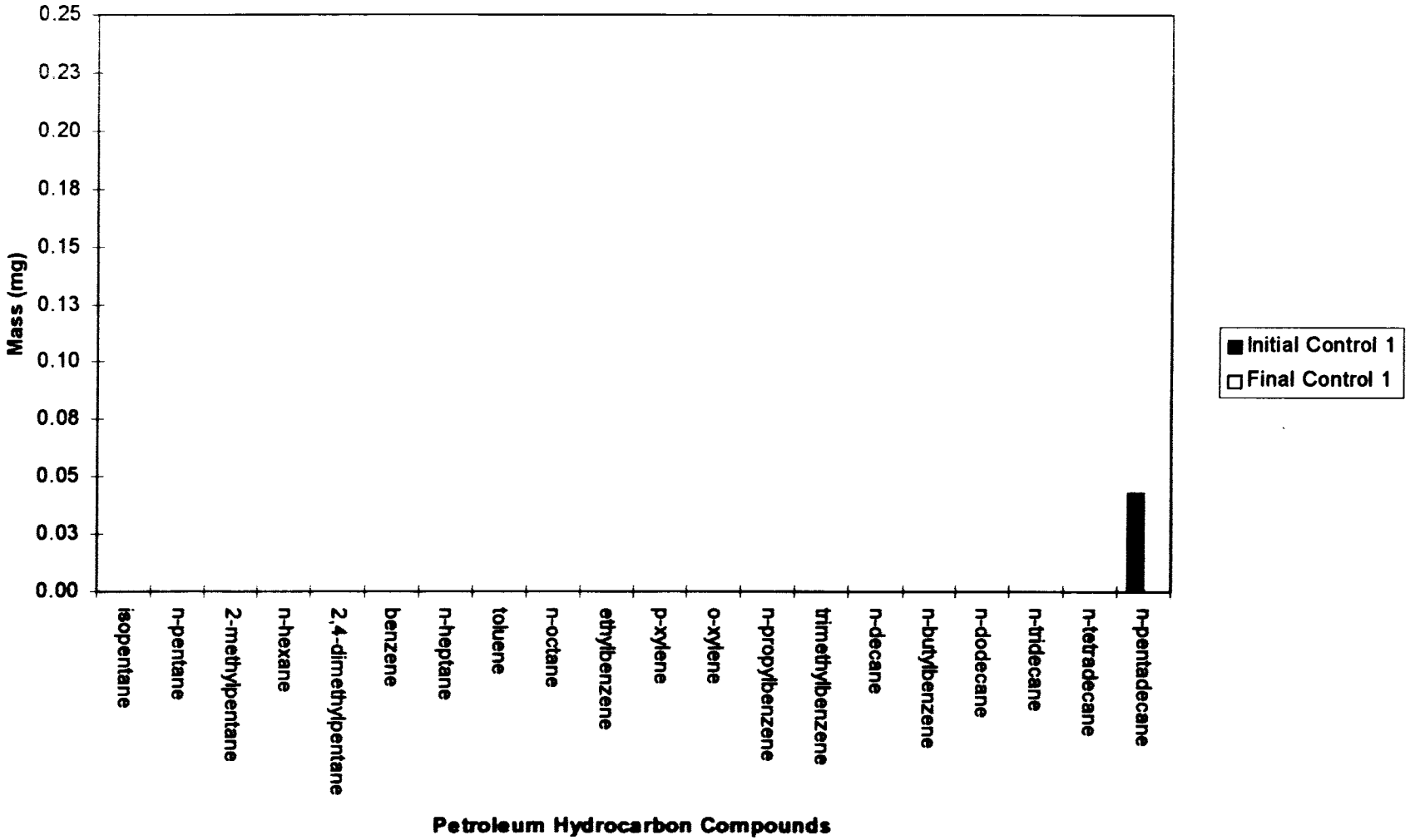

Figure 14. Initial and Average of the Final Mass of Petroleum Hydrocarbon Compounds (mg) in Reactors Containing Uncontaminated Soil During Experiment #1.

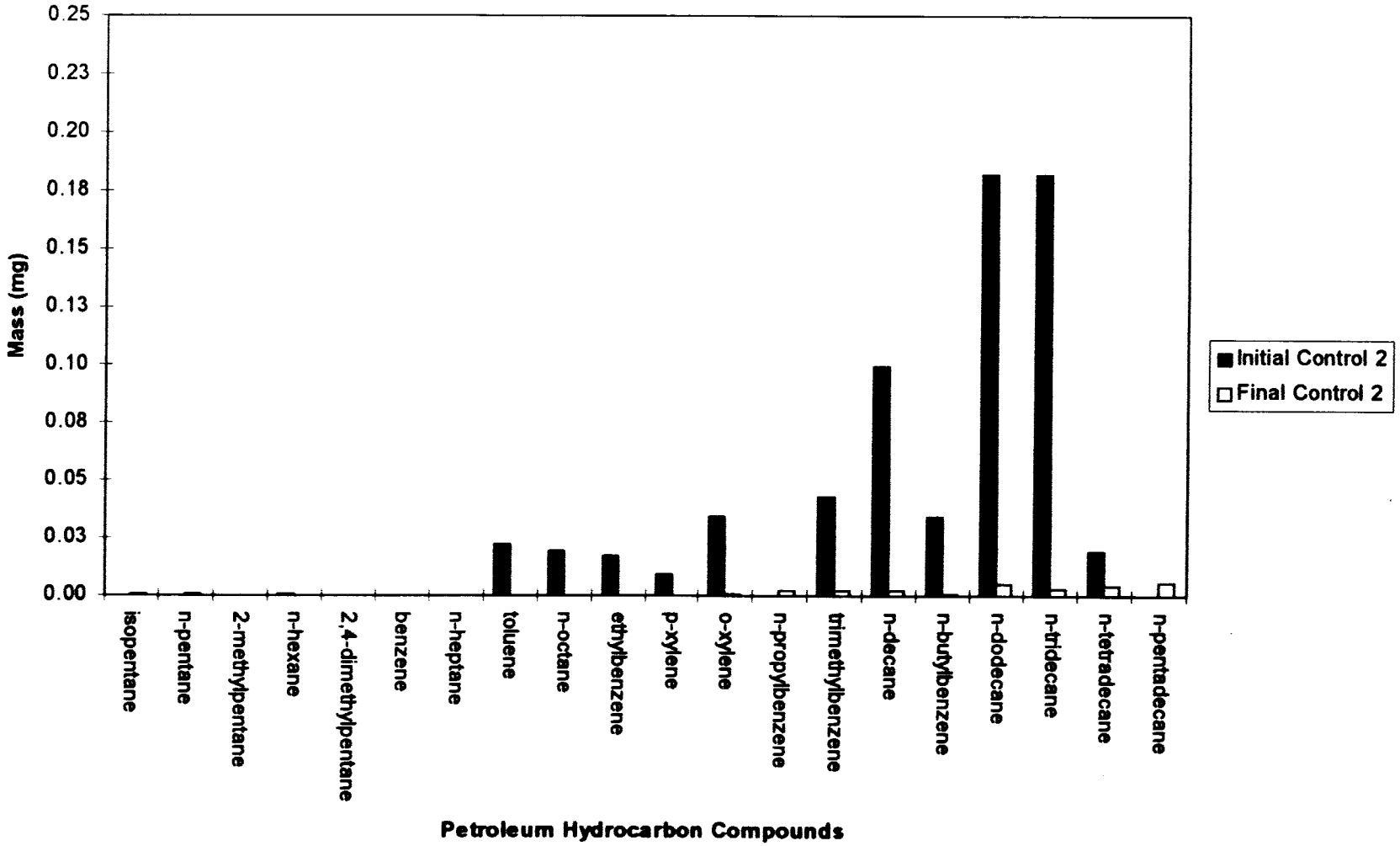

Figure 15. Initial and Final Mass of Petroleum Hydrocarbon Compounds (mg) in Reactors Containing Contaminated Soil with Sterilized Amendment During Experiment #1.

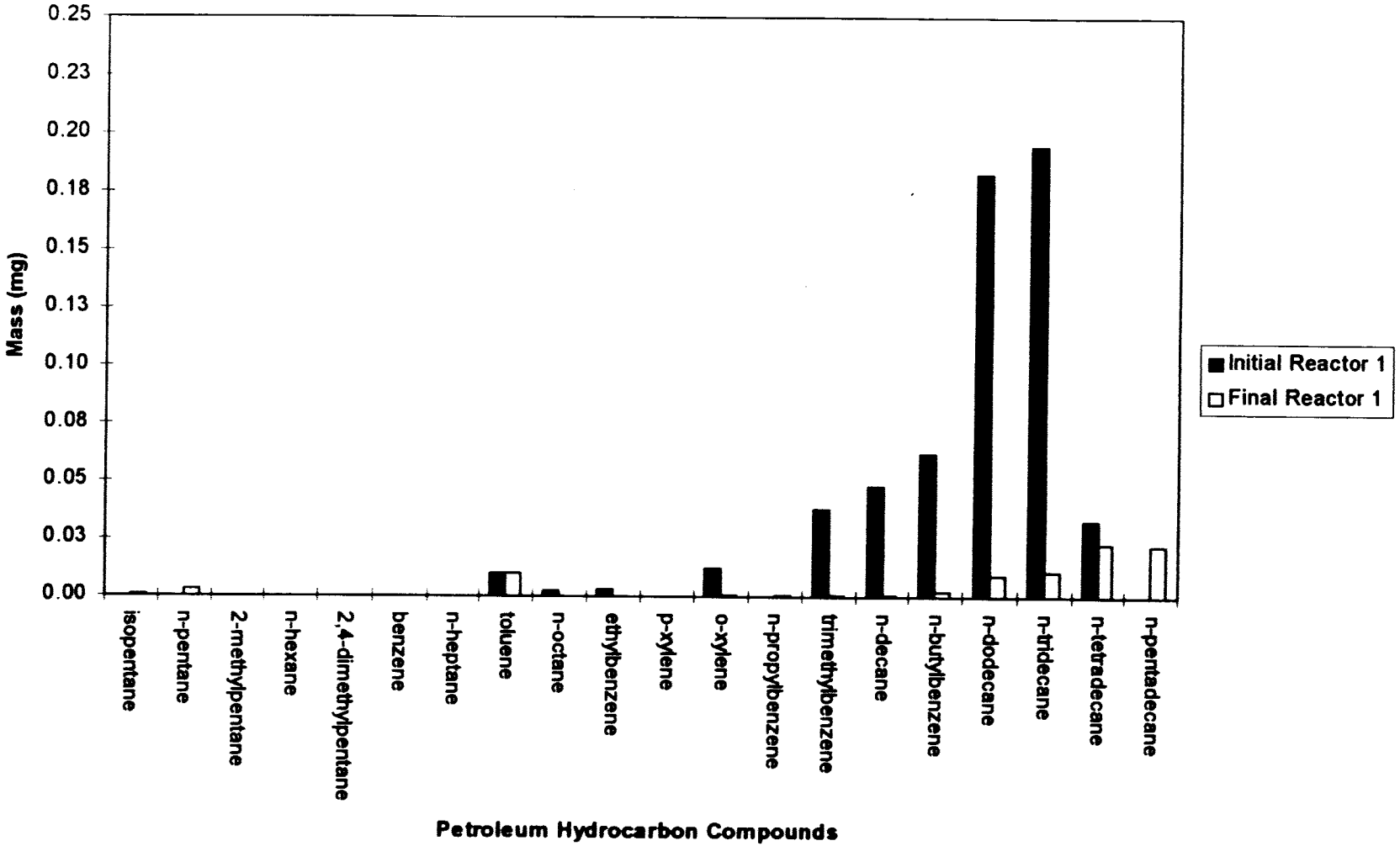

Figure 16. Initial and Average Final Mass of Petroleum Hydrocarbon Compounds (mg) in Reactors Containing Contaminated Soil During Experiment #1.

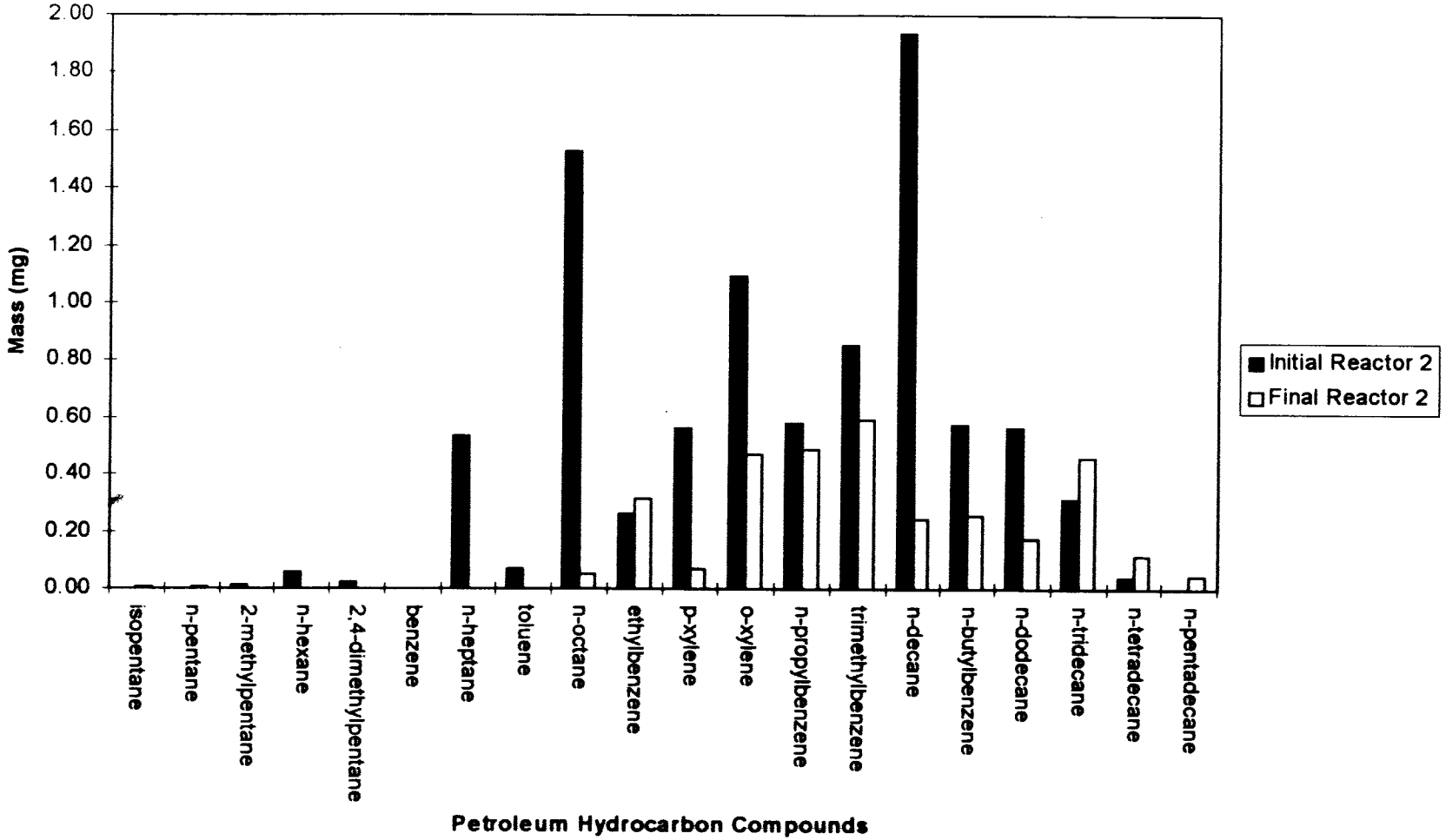

Figure 17. Initial and Average Final Mass of Petroleum Hydrocarbon Compounds (mg) in Reactors Containing Uncontaminated Soil with Amendment and JP-4 Addition During Esperiment #1. (Note: Y-axis is greater than those of that of the other experimental conditions)

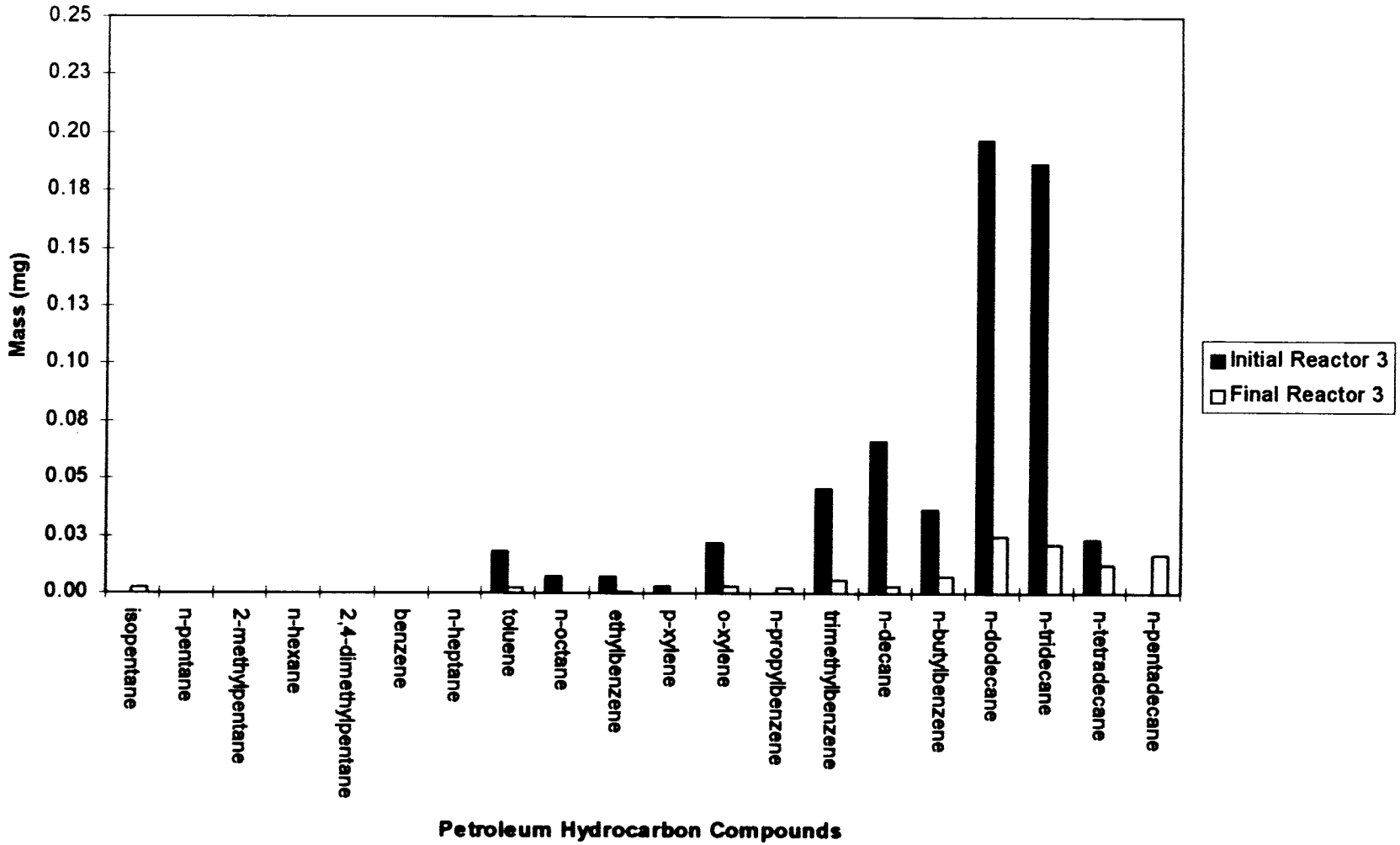

Figure 18. Initial and Average Final Mass of Petroleum Hydrocarbon Compounds (mg) in Reactors Containing Contaminated Soil with Microbial Amendment During Experiment #1.

Figure 18 illustrates the mass of petroleum hydrocarbon compounds in the contaminated soil with the microbial amendment addition (Reactor 3). All hydrocarbons were decreased except *n*-pentadecane. The *n*-pentadecane was not present at the beginning of the experiment but was detected at the end of the experiment.

4.3.4 Soil Moisture

The results for the soil moisture analyses conducted during Experiment #1 are presented in Table 5. Each of the five experimental conditions resulted in an average net loss of moisture from the reactor system after the 60-day incubation period. Because each reactor was set up as a sealed system, the loss of moisture was attributed to the evaporation that occurred during atmospheric exchanges throughout the incubation period.

The greatest moisture loss occurred in the uncontaminated soil with the amendment and JP-4 addition, where the average net loss was 30.8%. The evaporative loss of moisture in these reactors may have been facilitated by hydrophobic interactions of JP-4 and soil water. All other net losses of moisture were less than seen in the fresh JP-4 condition, with the slightest moisture loss occurring in the uncontaminated control. Here the average moisture loss was 10.8%.

The impact of this moisture loss in all five conditions on the activity of the present microbiological population was most likely minimal. However, this analysis in combination with others, such as microbiological enumerations or dehydrogenase activities testing, would support this claim in a microbiological amendment evaluation.

4.3.5 Soil pH

The results of soil pH measurements made before and after the 60-day incubation are presented in Table 6. The soil pH resulted in a slight increase for each of the five experimental conditions by the end of the incubation period. The pH unit changes for all conditions, except the condition of uncontaminated soil with amendment and JP-4 addition, were very similar. The dissimilar condition, however, resulted in an increase that was approximately 2.5 times greater than for the other conditions. The greater loss of water in this condition over the incubation period, as seen in Table 6, may have contributed to this slightly greater pH value. However, the final pH in all conditions remained within a suitable range for bacterial enzymatic reactions and nutrient bioavailability.

Table 5. Results from Soil Moisture Analysis Conducted on Soil Samples During Experiment # 1.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	18.29	15.44	16.42	17.46	
2	17.75	15.60	16.62	16.65	
3	18.39	15.60	16.44	15.39	
Average	18.14	15.55	16.49	16.50	-10.81
Contaminated Soil with No Amendment					
1	21.21	17.86	16.11	14.66	
2	21.39	18.85	16.04	15.00	
3	20.56	18.95	16.08	15.32	
Average	21.05	18.55	16.08	14.99	-21.43
Contaminated Soil with Sterilized Amendment Addition					
1	21.46	NS	15.92	15.28	
2	21.76	NS	16.17	15.48	
3	21.58	NS	16.89	16.21	
Average	21.60	0.00	16.33	15.66	-25.97
Contaminated Soil with Amendment Addition					
1	21.99	16.68	15.29	15.74	
2	20.63	18.98	15.81	16.04	
3	20.29	19.45	16.45	16.61	
Average	20.97	18.37	15.85	16.13	-19.97
Uncontaminated Soil with Amendment and JP-4 Addition					
1	17.59	11.93	11.89	12.31	
2	17.80	12.00	12.23	12.55	
3	18.02	12.38	12.89	12.72	
Average	17.80	12.10	12.34	12.52	-30.78

Table 6. Results from pH Analysis Conducted on Soil Samples During Experiment #1.

Conditions	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Final Average	pH Change
Uncontaminated Soil with No Amendment	6.29	6.70	6.73	6.82	6.75	0.46
Contaminated Soil with No Amendment	6.95	7.49	7.52	7.54	7.52	0.57
Contaminated Soil with Sterilized Amendment Addition	7.11	NS	7.51	7.60	7.56	0.45
Contaminated Soil with Amendment Addition	6.91	7.84	7.48	6.89	7.40	0.49
Uncontaminated Soil with Amendment and JP-4 Addition	6.25	7.46	7.55	7.60	7.54	1.29

4.3.6 Dehydrogenase Activity in Soil Samples

The results for dehydrogenase activity measurements made in Experiment #1 are presented in Table 7. The average percent change for each of the experimental conditions does not appear to be significant. A single bacterial cell contains approximately 1,000 enzymes. Although only a fraction of this enzyme pool would be dedicated to dehydrogenase activities, and thus would be detected in this assay, it was expected that after a 60-day incubation period the results would indicate final values in orders of magnitude greater than the initial soil measurements. However, this did not appear to be the case. In fact, in two of the five experimental conditions (contaminated soil with sterilized amendment addition and contaminated soil with amendment addition), the final detected values were less than their respective initial values. In these two conditions the percent average change was within the range of error between triplicate reactors and, therefore, dehydrogenase activity comparisons between these experimental conditions could not be made with any certainty.

In addition, the filtration step associated with this analysis was awkward and time consuming. The cotton filter may have adsorbed some of the TPF and resulted in the loss of the dehydrogenase indicator. Such a loss would contribute to a false negative reading and increase the margin of experimental error.

4.3.7 Microbial Enumerations in Soil Samples

Table 9 shows the microbial numbers for the first experimental run. A negative average change indicates that the final number of microbes is less than the initial microbial count. In all the conditions except one the microbial numbers decreased. Because this experiment ran for 60 days, the nutrients available to the microbes may have been limited, causing the number to decrease. The contaminated soil with microbial amendment added nutrients as well as microbes to the contaminated soil. These added nutrients helped the microbes to thrive. The uncontaminated soil with the microbial amendment and JP-4 addition had a ten-fold decrease in microbes. The mass of petroleum hydrocarbons was much greater in this condition than in any other condition (see Table 4, Section 4.3.3), and thus inhibited the growth of either the indigenous microbes or the amendment microbes, or perhaps both.

Table 7. Results from Dehydrogenase Activity Analysis (µg-H/g-dry soil) Conducted on Soil Samples During Experiment #1.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	1.22e+02	8.41e+02	2.37e+02	2.29e+02	
2	6.12e+01	2.36e+02	3.65e+02	4.01e+02	
3	6.12e+01	1.77e+02	7.17e+02	3.47e+02	
Average	8.15e+01	4.18e+02	4.40e+02	3.26e+02	384.18
Contaminated Soil with No Amendment					
1	2.51e+02	7.32e+02	2.87e+03	8.21e+02	
2	4.39e+02	6.62e+02	9.14e+02	4.63e+02	
3	3.76e+02	6.06e+02	5.90e+02	8.73e+02	
Average	3.55e+02	6.67e+02	1.46e+03	7.19e+02	166.76
Contaminated Soil with Sterilized Amendment Addition					
1	2.14e+03	NS	1.00e+03	5.27e+02	
2	1.64e+03	NS	4.01e+02	5.22e+02	
3	1.83e+03	NS	3.57e+02	4.13e+02	
Average	1.87e+03	0.00e+00	5.86e+02	4.87e+02	-71.30
Contaminated Soil with Amendment Addition					
1	1.69e+02	8.59e+02	6.00e+02	3.47e+02	
2	1.32e+03	4.91e+02	2.37e+02	3.47e+02	
3	1.32e+03	2.38e+02	3.39e+02	4.60e+02	
Average	9.36e+02	5.29e+02	3.92e+02	3.85e+02	-53.51
Uncontaminated Soil with Amendment and JP-4 Addition					
1	2.44e+02	3.43e+02	3.46e+02	3.96e+02	
2	2.44e+02	1.69e+02	4.03e+02	2.34e+02	
3	3.05e+02	4.44e+02	4.00e+02	3.90e+02	
Average	2.64e+02	3.19e+02	3.83e+02	3.40e+02	31.36

4.3.8 Nutrient Concentrations in Soil Samples

The results for soil nutrients and cation exchange capacity are presented in Table 8. The two nutrients of primary importance for soil microorganisms are nitrogen and phosphorus. Nitrogen is an important constituent in cellular amino acids and in the nucleic acids DNA and RNA which contain the genetic codes for bacterial function and reproduction. Phosphorus is an important constituent of proteins and adenosine triphosphate (ATP), a molecule that is important for the storage and transfer of chemical energy. Inadequate quantities of these two elements could be detrimental to population growth and subsequently to contaminant degradation.

The results presented in Table 8 were used to determine several nutrient parameters. First and most importantly, the analysis provided information as to whether there were sufficient amounts of key nutrients in the soil prior to incubation. The results presented in Table 8 indicated that this was the case in all five experimental conditions and that the soil microorganisms should not have been nutrient-limited during the early incubation period.

Second, the analytical results provided information as to whether or not important soil nutrients had become limited by the time that the experiment was terminated, and whether or not any nutrient reduction may have been significant enough to have an impact on microbial activity. The results indicated that for all five experimental conditions the final values for nutrient concentrations did not vary significantly from their respective initial values. Therefore, any growth inhibition that may have occurred during the course of incubation should not be related to nutrient concentrations.

Third, this analysis provided background nutrient concentration data. The soil used in these experimental runs seemed to have ample nutrients already available as indicated by the high nutrient concentrations in the control soil. However, when using different soil types for experimentation, nutrient limitations most likely will persist.

Finally, the analytical results provided valuable dosing information. The nutrient concentrations within the microbiological amendment were not known before or after dilution of the material. The amendment nutrient concentrations were not learned until after the amendment had been prepared and added to the soil, and subsequently sent out for initial analysis. The data presented in Table 8 indicate that addition of the nutrient formulation at the suggested dose did not produce any increase in nutrients over the background concentrations of any of the five experimental conditions.

Table 8. Results of Cation Exchange Capacity and Nutrient Analyses Conducted on Soil Samples During Experiment #1.

Identification	CEC (meq/100 g)	N (ppm)	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	Na (ppm)
Uncontaminated Soil with No Amendment							
Final Reactor A	3.9	393	35	23	710	22	16
Final Reactor B	3.9	385	25	22	710	20	12
Final Reactor C	4.8	369	24	20	920	17	10
Final Avg.	4.2	382	28	22	780	20	13
Avg. Change (%)	35	-8	12	-25	24	-21	58
Contaminated Soil with No Amendment							
Initial	3.0	297	8	24	1,120	28	14
Final Reactor A	8.3	336	5	25	1,580	26	16
Final Reactor B	6.1	290	3	29	1,170	19	14
Final Reactor C	6.6	310	26	29	690	20	15
Avg.	7.0	312	11	28	1,146	22	15
Avg. Change (%)	133	5	42	15	2	-23	7
Contaminated Soil with Sterilized Amendment Addition							
Initial	3.0	314	10	25	1,100	25	10
Final Reactor A	NS	NS	NS	NS	NS	NS	NS
Final Reactor B	6.7	300	6	29	1,280	20	14
Final Reactor C	6.1	280	40	20	1,170	20	14
Avg.	6	290	23	25	1,225	20	14
Avg. Change (%)	113	-8	130	-2	11	-20	40
Contaminated Soil with Amendment Addition							
Initial	3.1	320	9	22	940	22	9
Final Reactor A	7.0	310	5	22	1,310	36	18
Final Reactor B	8.4	300	6	22	1,610	26	17
Final Reactor C	7.4	306	7	29	1,410	21	17
Avg.	8	305	6	24	1,443	28	17
Avg. Change (%)	145	-5	-33	11	54	26	93
Uncontaminated Soil with Amendment and JP-4 Addition							
Initial	3.0	318	26	28	590	24	6
Final Reactor A	3.8	390	26	29	690	20	15
Final Reactor B	5.6	415	25	21	1,050	24	18
Final Reactor C	4.5	345	23	20	840	22	15
Avg.	5	383	25	23	860	22	16
Avg. Change (%)	54	21	-5	-17	46	-8	167

NS No sample due to the loss of this reactor during incubation.

Table 9. Results of Microbial Enumerations Conducted on Soil Samples During Experiment #1

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	6.38×10^5	5.16×10^5	4.84×10^5	7.55×10^5	
2	7.09×10^5	4.55×10^5	4.68×10^5	3.88×10^5	
3	5.79×10^5	5.53×10^5	4.39×10^5	4.90×10^5	
Average	6.42×10^5	5.08×10^5	4.64×10^5	5.44×10^5	-21.29
Contaminated Soil with No Amendment					
1	9.97×10^4	2.55×10^5	9.30×10^4	9.95×10^4	
2	1.17×10^5	9.34×10^4	1.01×10^5	6.67×10^4	
3	1.41×10^5	9.68×10^4	8.55×10^4	8.00×10^4	
Average	1.19×10^5	1.48×10^5	9.31×10^4	8.21×10^4	-9.47
Contaminated Soil with Sterilized Amendment Addition					
1	1.62×10^5	NA	7.29×10^4	1.14×10^5	
2	1.06×10^5	NA	1.10×10^5	1.34×10^5	
3	1.23×10^5	NA	1.55×10^5	1.29×10^5	
Average	1.30×10^5	NA	1.12×10^5	1.29×10^5	-7.31
Contaminated Soil with Amendment Addition					
1	1.38×10^5	1.35×10^5	1.15×10^5	2.45×10^5	
2	1.20×10^5	9.21×10^4	1.26×10^5	1.38×10^5	
3	1.08×10^5	1.01×10^5	9.92×10^4	2.04×10^5	
Average	1.22×10^5	1.09×10^5	1.13×10^5	1.96×10^5	14.21
Uncontaminated Soil with Amendment and JP-4 Addition					
1	1.74×10^6	1.13×10^5	1.38×10^5	1.19×10^5	
2	1.19×10^6	1.22×10^5	1.30×10^5	1.15×10^5	
3	1.76×10^6	8.51×10^4	1.40×10^5	1.06×10^5	
Average	1.56×10^6	1.07×10^5	1.36×10^5	1.13×10^5	-92.39

4.3.9 Carbon in Soil Samples.

The results for the inorganic and organic carbon analyses conducted using the UIC methods are presented in Table 10 and Table 11, respectively. The positive percent average change values associated with this table indicate an increase in inorganic carbon at the time of reactor harvesting. The results for inorganic carbon analysis conducted in Experiment #1 indicate a net increase in inorganic carbon for each of the five experimental conditions.

The resulting increase in inorganic carbon most likely occurs due to the accumulation of carbon dioxide, which is produced during microbiological respiration. The accumulation of CO_2, which is either incorporated into the carbonate system or complexed with various soil elements such as calcium, would increase the inorganic content of the soil. The CO_2 remaining in the free gaseous phase eventually would be removed from the reactor system during atmospheric exchanges.

The results presented in Table 10 indicate that some absorption or complexation had taken place in each of the five experimental conditions, as an overall increase in inorganic content was observed. However, there does not appear to be any relationship regarding these increases among the five experimental conditions. The greatest increase of inorganic carbon occurred in the uncontaminated control. Although soil moisture and pH have an impact on the fate of inorganic carbon, it is unlikely that the insignificant differences in these parameters among the various experimental conditions contributed to the scattered inorganic carbon results. It was anticipated that the uncontaminated control would have resulted in the lowest inorganic carbon concentrations and, for those experimental conditions where viable microbial populations were present, the inorganic carbon concentrations would be greater.

The reported results for this analysis are difficult to explain and may be due to the methodological approach. The UIC carbon analyzer used for this method allows for a maximum sample size of approximately 200 mg. This volume is often difficult to work with, and one can never be certain that a representative sample has been collected because any slight variability in soil moisture content or the inclusion and exclusion of larger soil particles, such as small rocks, could skew the results. However, the sample size could not be increased beyond 200 mg, and the potential impact of the loading size on the analytical results could not be further investigated due to the limitations of the UIC instrument.

The results of the organic carbon analyses conducted for Experiment #1 are presented in Table 11. Again, there does not appear to be any relationship among the five different experimental conditions. It was expected that organic carbon would be removed more readily from those experimental conditions which supported a viable microbial population. The results presented in Table 11 depict a situation where greater

Table 10. Results of Inorganic Carbon Analysis (ppm) Conducted Using the UIC Method on Soil Samples During Experiment #1.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	198	1765	1094	1465	
2	226	970	2300	1013	
3	245	1524	1080	1346	
Average	223	1420	1491	1275	525
Contaminated Soil with No Amendment					
1	164	364	328	333	
2	642	615	498	284	
3	155	276	362	510	
Average	320	419	396	375	24
Contaminated Soil with Sterilized Amendment Addition					
1	235	NS	1371	1100	
2	187	NS	790	920	
3	279	NS	1156	NS	
Average	234	0	1106	1010	353
Contaminated Soil with Amendment Addition					
1	265	478	235	256	
2	226	353	674	254	
3	244	549	629	240	
Average	245	460	513	250	66
Uncontaminated Soil with Amendment and JP-4 Addition					
1	274	706	282	948	
2	269	1093	270	419	
3	174	334	235	220	
Average	239	711	262	529	110

Table 11. Results of Organic Carbon Analysis (ppm) Using the UIC Method on Soil Samples During Experiment #1.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	8004	7606	7547	7603	
2	7770	7807	7943	7440	
3	8185	7074	7601	7706	
Average	7986	7496	7697	7583	-4.94
Contaminated Soil with No Amendment					
1	4398	5028	5536	333	
2	5864	10655	5526	284	
3	5535	4135	6323	510	
Average	5266	6606	5795	375	-19.12
Contaminated Soil with Sterilized Amendment Addition					
1	4400	NS	5327	5478	
2	4745	NS	5930	5788	
3	5430	NS	5882	6459	
Average	4858	0	5713	5908	19.60
Contaminated Soil with Amendment Addition					
1	4844	5101	6349	6166	
2	5113	5181	5838	6191	
3	5720	5043	5583	5760	
Average	5226	5108	5923	6039	8.88
Uncontaminated Soil with Amendment and JP-4 Addition					
1	7716	8002	8087	8221	
2	9817	7764	8159	7913	
3	11028	8017	7660	10763	
Average	9520	7928	7969	8966	-12.95

organic carbon removal occurs in the uncontaminated and contaminated control, as evidenced by the negative percent average change values for these two experimental conditions.

The sample preparation for organic carbon analyses is identical to the methods mentioned above for inorganic carbon. Therefore, a similar sample size is used for this analysis. As previously mentioned, it is not certain whether this small sample is representative of true conditions. Often roots and other soil organic matter are encountered during the sample loading procedure. Random appearance of these types of materials in the sample will result in an increased organic load and biased data. The ability to increase the sample would ensure a more homogeneous sample and would help to obtain more meaningful results.

4.3.10 Particle Size Distribution

The characterization results for the soil used in the protocol development is presented in Table 12. The results of the analyses performed by A&L Laboratories, Inc. indicated that the soil was 96.4% sand composed of mostly of medium and fine particles.

4.3.11 Cation Exchange Capacity

The results of the cation exchange capacity analyses, conducted for Experiment #1, are presented along with the Experiment #1 nutrient availability data in Table 8.

Table 12. Results of Particle Size Distribution and Textural Analyses Conducted on Soil Samples During Experiment #1.

Soil Characterization	
Soil Classification	Sand
Sand (%)	96.4
Silt (%)	2.3
Clay (%)	1.3
Particle Size Distribution	
Very Coarse Sand (1-2 mm) (%)	0.1
Coarse Sand (0.5-1.0 mm) (%)	0.5
Medium Sand (0.25-0.5 mm) (%)	50.6
Fine Sand (0.10-0.25 mm) (%)	44.8
Very Fine Sand (0.05-0.10 mm) (%)	0.4

5.0 EXPERIMENT #2

The second round of experiments was conducted incorporating some key changes in the methods used in the first experiment and described in the Experimental Design Test Plan (Battelle, 1994). The column reactors were set up in triplicate under the five different experimental conditions as described for the first experiment. One of the key changes was modifying the inoculum to increase the bacterial inoculation rate by 100 times the rate of Experiment #1. This was necessary to better evaluate the sensitivity of the analytical protocols. Some modifications were made to the analytical protocols as well, either to reduce waste production or to increase sensitivity. These modifications are described in the following sections.

5.1 Experimental Methods

Although the experimental methods used during the second experiment for processing the soils, setting up the column reactors, operating and monitoring the reactors, and harvesting the reactors at the end of the experimental run were based on the methods described for the original experiment, some modifications were made to improve specific methods. The sections below indicate whether the methods remained the same or required modification. When a method was modified, the reason for the modification is provided and a detailed description of the modified method is presented.

5.1.1 Soil Processing

The soil processing procedure described in Section 4.1.1 was unchanged and was used for this experiment.

5.1.2 Reactor Design and Setup

The reactor design for Experiment #2 was similar to the design established in Experiment #1, with only slight modifications. In this experiment, 15 column-type reactors were configured and filled with 87 g of wet soil. The 15 reactors represented triplicate sets of each of the five experimental conditions. Glass wool was placed at each of the end caps to separate the soil/Teflon™-cap interface. The use of glass wool was intended to help limit the loss of moisture during atmospheric exchanges and to eliminate plugging of the sample line by soil particles. After the soil was loaded into the reactors, the end caps were fastened and the columns were mounted to a floor rack. The water jackets of each of the column reactors were connected with

Tygon™ tubing so that all of the reactors were in series. The tubing was plumbed to a temperature-controlled circulating water bath capable of both heating and cooling as explained for Experiment #1.

The reactors were incubated for 30 days at a temperature of 25°C. The influent temperature of the water bath was monitored daily to ensure that the temperature of the water leaving the reactors was maintained. The atmospheres of each reactor were exchanged routinely throughout the incubation period with the reactors in place, as explained in Section 4.1.3. After the 30-day incubation period, the reactors were harvested for analyses on the incubated soils.

5.1.3 Reactor Operation and Monitoring

5.1.3.1 Maintaining Temperature. The columns were kept at a constant temperature by recirculating water from a constant-temperature water bath as described in Section 4.1.3.1.

5.1.3.2 Atmosphere Exchanges. Gas exchange of 100 mL occurred every 4 days or less depending upon the oxygen measurements. After reviewing the procedure for exchanging gases, it was believed that the entire column was being evacuated. Minor modifications on opening and closing the valves were made for the sampling process. After the lab air bag and the desiccator with sampling bag were in place on the column, the valve on the top of the column was opened first, followed by the valve on the bottom of the column. Next, the clean air valve was opened and the first valve (valve 1) on the desiccator was opened last. Valve 1 on the desiccator was immediately closed when the 100 mL of air was depleted from the clean air Tedlar™ bag. One sample series was run according to this procedure, yet after reviewing the process it was thought a vacuum was being pulled on the columns. For the final three sample series, a new method was used to avoid placing a vacuum on the columns.

With the desiccator and air bag assembled onto the columns, valve 1 on the desiccator, the top valve of the column, and the bottom valve on the column were opened, respectively. The air bag was opened slowly until all the air was expelled from the bag. The valves on the air bag and on the bottom of the column were closed. Valve 2 of the desiccator was opened to allow the desiccator and the column to equilibrate to atmospheric pressure. The valve at the top of the column was closed, followed by valve 1 of the desiccator. After the sixth sample series, an electric pump was used to evacuate the desiccator instead of the 1,000-mL syringe that had been used previously.

5.1.3.3 **Monitoring Respiration.** The monitoring conditions and procedures used during this experimental run were the same as those used in the first experimental run as described in Section 4.1.3.3.

5.1.4 Reactor Harvesting

After the 30-day incubation period it was necessary to remove the mounted column reactors from the floor rack to conduct further testing. The water cooling system was shut off and the Tygon™ tubing of the cooling circulation unit was removed as explained in Experiment #1. The reactors were all harvested in an identical manner following the same procedure as explained in Experiment #1. The only exception to this procedure was that no soil was collected and sent to an outside laboratory because it was decided that nutrient analyses would not be performed.

5.2 Analytical Methods

All of the analytes monitored in Experiment #1 with the exception of the nutrients and soil physical properties were monitored in Experiment #2. The analytes omitted during this experiment were constant between experiments, so analysis was not necessary. Their omission does not mean that these protocols were unimportant or that they were considered for exclusion from the final protocol. Most of the analytical methods used in the second experimental run were the same as in the first experiment. However, several of the methods were modified to either reduce the amount of hazardous waste produced by the method and/or to increase the sensitivity of the analysis. The following sections indicate whether the methods remained the same or were modified. In the event that modifications were made, the revised protocol is described.

5.2.1 Oxygen and Carbon Dioxide in Gas Samples

Oxygen and carbon dioxide concentrations were measured in gas extracted from the reactors during the atmospheric flushing procedures according to the methods used in Experiment #1 as described in Section 4.2.1. No modifications were made to this method.

5.2.2 Petroleum Hydrocarbons in Gas Samples

Petroleum hydrocarbon concentrations were measured in gas extracted during the atmospheric flushing procedures according to the method used during Experiment #1 as described in Section 4.2.2. No modifications were made to this method.

Step 1. Prepare a 3% TTC solution by dissolving 3 g of 2,3,5-TTC in 80 mL of high-performance liquid chromatography (HPLC)-grade water then adjusting the volume to 100 mL.

Step 2. Prepare a TPF standard solution by adding 100 mg of TPF in about 80 mL of methanol then adjusting the volume to 100 mL. Add a magnetic stir bar to this solution and allow to mix for approximately 1 hour. This will result in a saturated stock solution of TPF.

Step 3. Prepare a set of calibration standards by transferring 1 mL of the saturated TPF solution into a 10-mL volumetric flask and bring the volume up to 10 mL with methanol. Pipet 50-µL, 200-µL, 500-µL, 1-mL, 1.5-mL, and 2-mL aliquots of this solution into 10-mL volumetric flasks and bring the volumes up to 10 mL with methanol to prepare standards of 0.5, 2.0, 5.0, 10.0, 15.0, and 20.0 µg of TPF/mL, respectively. Measure the intensity of the reddish color on a spectrophotometer at a wavelength of 485 nm. Generate a calibration curve by plotting the absorbance readings against the concentration of TPF.

Step 4. Thoroughly mix 10 g of the sample soil with 0.1 g of $CaCO_3$ in a 20-mL glass scintillation vial. Transfer 3 g of this mixture into each of three labeled, 16 × 100 mm screw-cap glass test tubes. Add 0.5 mL of the 3% TTC solution and 1.25 mL of HPLC-grade water to each test tube. Cap each tube and mix by vortexing for 15 seconds. Place each test tube in an incubator for 48 hours at 37°C.

Step 5. Following incubation, add 5 mL of HPLC-grade methanol to each test tube. Vortex each test tube for approximately 30 seconds. Centrifuge each test tube at 50 rpm for 5 minutes. Collect the extract from each tube by pouring off the supernatant into a clean, dry, 25 × 100 mm glass, screw-cap test tube. Repeat this procedure four additional times, collecting the accumulative extract for each soil sample.

Step 6. This step is optional. Depending on the soil type, a small quantity of excessive fines may not settle out during centrifugation at low-rpm values. If this is the case, a small aliquot of the accumulative extract may be transferred to a microcentrifuge tube and centrifuged at greater rpm values (i.e. ~ 2,000 rpm) to separate the soil from the extract before proceeding to Step 7.

Step 7. Remove an aliquot of the cumulative extract produced in Step 5 and/or Step 6. Measure the intensity of the reddish color using a spectrophotometer at a wavelength of

485 nm and determine the amount of TPF produced by comparison against the calibration curve prepared in Step 3.

5.2.7 Microbial Enumerations in Soil Samples

The microbial enumeration procedure described in Section 4.2.7 was modified to increase staff efficiency and to avoid the potential for contamination associated with the use of paper toweling as the absorbent material. The modifications to the method were as follows.

1. **Dilution Tubes**. Used a phosphate buffer solution in the dilution tube in place of the minimal salts media. This resulted in a significant time and cost savings as the number of ingredients required is drastically reduced. The dilution tubes were prepared by making 0.2M solutions of K_2HPO_4 and KH_2PO_4 in distilled water. The solutions were mixed in the ratio 77 parts K_2HPO_4 to 28 parts KH_2PO_4 and the pH was adjusted to 7.2 with KOH and/or HCl. The resulting potassium phosphate buffer solution was dispensed into test tubes as before in 10-mL and 9-mL aliquots.
2. **JP-4 Addition to Plates and on Top of Plates.** Added the 10 µL of JP-4 jet fuel to the basal inorganic medium with a 100-µL pipet instead of a gastight syringe. Pipetted 100 µL of JP-4 jet fuel onto a sterilized Gelman 47-mm absorbent pad instead of a sheet of paper toweling, placed the pad on top of the petri dishes inside the plastic bag, and sealed the bags with colored marking tape.

5.2.8 Nutrient Concentrations in Soil Samples

Nutrient analyses for Experiment #2 were not conducted. It was assumed that the nutrient concentrations would be similar to the concentrations obtained for Experiment #1, because the same nutrient addition protocol was applied for this experiment.

5.2.9 Carbon in Soil Samples

Organic and inorganic carbon contents were measured in the soil samples for each experimental condition, and from each reactor after harvesting, using the method on the UIC Model 5012 Carbon Analyzer as described for Experiment #1 (Section 4.2.9). Although no modifications were made to this method, an additional method for conducting organic matter analysis was examined to determine if this method was a more efficient and better way to measure the organic content of the soils. The added method includes

gravimetric determination of the organic matter following combustion. The additional method was coupled with the moisture determination procedure and conducted according to the following steps:

Step 1. Turn on the drying oven and allow the temperature to equilibrate at 105°C.

Step 2. Preweigh and label drying dishes.

Step 3. Weigh out a 5-g aliquot of soil in triplicate and place in the preweighed, labeled drying dish.

Step 4. Place dishes containing the soil sample into the drying oven and allow them to dry for 24 hours.

Step 5. Remove dishes and place them into a desiccator at room temperature. Allow the sample dishes to cool to constant weight at room temperature.

Step 6. Remove the sample dishes from the desiccator and record the cumulative weight of the dish plus the sample after drying.

Step 7. Turn on the muffle furnace and allow the temperature to equilibrate at 550°C.

Step 8. Place the sample dishes in the muffle furnace, close the door, then keep the samples in the muffle furnace for 1 hour to allow adequate time for complete combustion.

Step 9. After 1 hour remove the dishes from the muffle and immediately place the dishes into a desiccator at room temperature. Allow the sample dishes to cool to constant weight at room temperature.

Step 10. Remove the sample dishes from the desiccator and record the cumulative weight of the dish plus the sample after muffling.

5.2.10 Particle Size Distribution

Soil particle size distribution and percent sand, silt, and clay in soil analyses were not conducted during this experiment. Because the same soil was used as in Experiment #1 and nothing was done to alter the physical properties of the soil, it was concluded that these parameters remained consistent with the results obtained during Experiment #1. The analysis was not considered unimportant and was not dropped from consideration for the final protocol.

5.2.11 Cation Exchange Capacity

Cation exchange capacity analysis was not included in Experiment #2. Because nothing was done to the soil to alter the CEC, it was concluded that the CEC values obtained during Experiment #1 were valid. The analysis was not considered unimportant and was not dropped from consideration for the final protocol.

5.3 Results

The analytical protocols conducted during Experiment #2 as described in Section 5.2 were completed and the data were reduced and evaluated. The results from each protocol are presented in the following sections along with a discussion of the trends between experimental conditions, the added value of the data obtained using the specified protocols, and any method modifications required to enhance the data obtained.

5.3.1 Oxygen and Carbon Dioxide in Gas Samples

The cumulative oxygen consumption and carbon dioxide production data for the uncontaminated soil are shown in Figure 19. Control 1 A consuming a total of 20.86 mg of oxygen, had the least oxygen consumed and the least carbon dioxide produced over the 31-day experimental period. The largest jump in oxygen consumption was from day 10, when 4.04 mg was consumed, to day 13, when 10.02 mg of oxygen was consumed. Only 0.90 mg of carbon dioxide was produced on day 24. No carbon dioxide was detected during the first 20 days and no more was detected after day 24.

For Control 1 B, a total of 26.66 mg of oxygen was consumed. Day 3 to day 10 was the biggest jump, from 0.80 mg to 6.06 mg of oxygen consumed. The carbon dioxide produced on day 3 was 0.92 mg. No more carbon dioxide was produced until the sampling on the final day, when the cumulative production of carbon dioxide equaled 1.77 mg.

Control 1 C had the largest oxygen consumption and carbon dioxide production among the three columns with the uncontaminated soil. On day 20, the oxygen present was at 16.79%; therefore 6.01 g of oxygen was consumed. The final oxygen consumption was 29.50 mg. The carbon dioxide production started at 1.22 mg on day 3. The total raised to 2.12 mg on day 6 and stayed there for day 10. On day 13 the total was 2.95 mg and stayed there for the next 7 days. The total carbon dioxide produced was 3.77 mg and was reached on day 24 of the experiment.

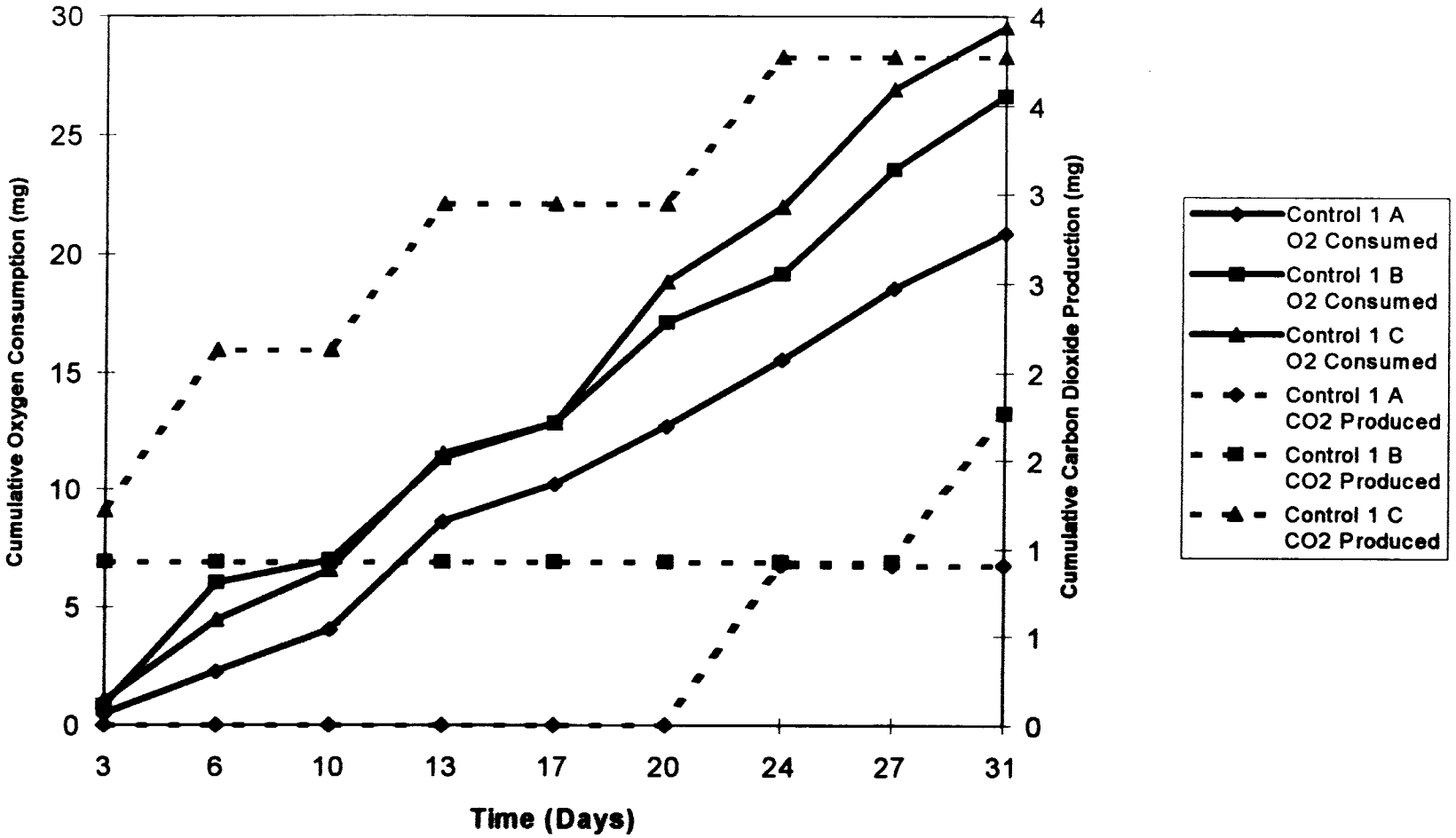

Figure 19. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production (mg) in Reactors Containing Uncontaminated Soil During Experiment #2.

Figure 20 shows the cumulative oxygen consumed and the cumulative carbon dioxide produced in reactors with the contaminated soil and sterilized amendment. The three columns exhibited steady increases of cumulative oxygen consumption and cumulative carbon dioxide produced. The contaminated soil amended with sterilized amendment consumed more oxygen and produced more carbon dioxide than the uncontaminated soil. The total oxygen consumed in Control 2 A was 55.16 mg. The total carbon dioxide produced was 40.15 mg. Control 2 B had the lowest amount of oxygen consumed and carbon dioxide produced among the three columns with contaminated soil and sterilized amendment. The total oxygen consumed was 43.83 mg and the total carbon dioxide produced was 38.42 mg. Control 2 C had the largest cumulative consumption of oxygen and production of carbon dioxide. The cumulative oxygen consumed was 70.73 mg and the cumulative carbon dioxide produced was 48.80 mg.

The data for cumulative oxygen consumed and carbon dioxide produced for the reactors containing only contaminated soil are shown in Figure 21. Reactor 1 B was lost due to leakage of water into the column. Reactor 1 A had less oxygen consumed than Reactor 1 C until day 20, when the gas sample from Reactor 1 A showed a value of 16.35% oxygen present, or 6.64 mg of oxygen consumed. Reactor 1 A had more oxygen consumed than Reactor 1 C. The oxygen consumed from Reactor 1 A was 28.50 mg and from Reactor 1 C was 25.89 mg. Reactor 1 C had more carbon dioxide produced than Reactor 1 A throughout the entire experiment. The cumulative carbon dioxide produced from Reactor 1 A was 15.50 mg, and from Reactor 1 C was 18.56 mg.

Figure 22 shows the cumulative oxygen consumed and the cumulative carbon dioxide produced from the uncontaminated soil with the microbial amendment and JP-4 addition. Reactor 2 B and Reactor 2 C were lost due to water leakage into the columns. Reactor 2 A had a steady increase in the carbon dioxide produced and the oxygen consumed. The production of carbon dioxide began to level off after day 20 of the experiment, adding approximately 1 mg at each of the final four samplings. The final production of carbon dioxide equaled 25.71 mg. The oxygen consumption totaled 33.97 mg.

Figure 23 shows a graph of the data for oxygen consumption and carbon dioxide production for the contaminated soil with the microbial amendment. Reactor 3 C was lost when water leaked into the column. Reactor 3 A had a steady climb of oxygen consumption. The higher consumption of oxygen between the two columns with contaminated soil and the microbial amendment was Reactor 3 A, with a total of 59.96 mg. Reactor 3 B had a total oxygen consumption of 45.53 mg. Reactor 3 A also had the higher production of carbon dioxide. Reactor 3 A had a total of 50.68 mg of carbon dioxide produced, whereas Reactor 3 B had 36.52 mg of carbon dioxide produced.

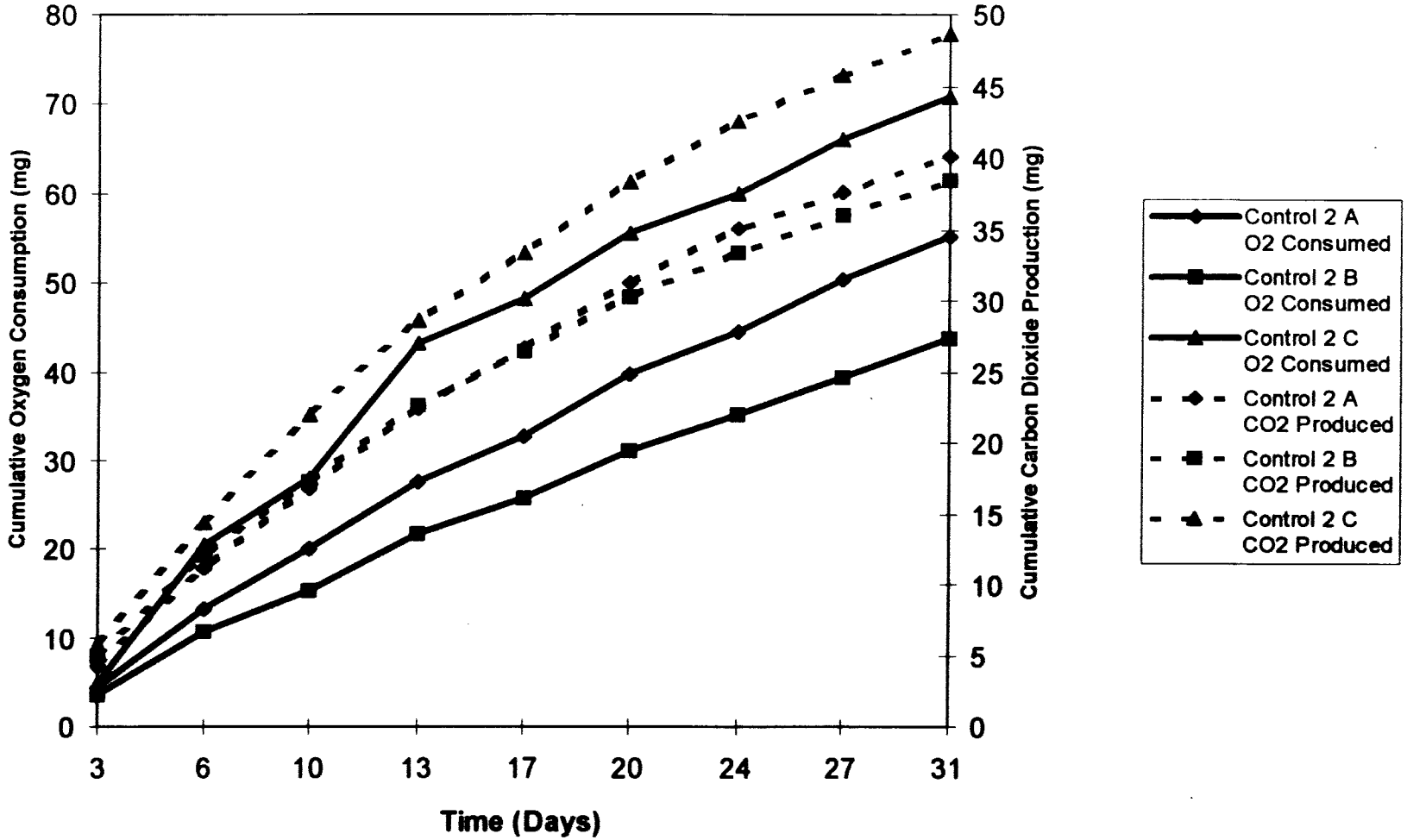

Figure 20. Cumulative Oxygen and Utilization and Cumulative Carbon Dioxide Production (mg) in Reactors Containing Contaminated Soil with Sterilized Amendment During Experiment #2.

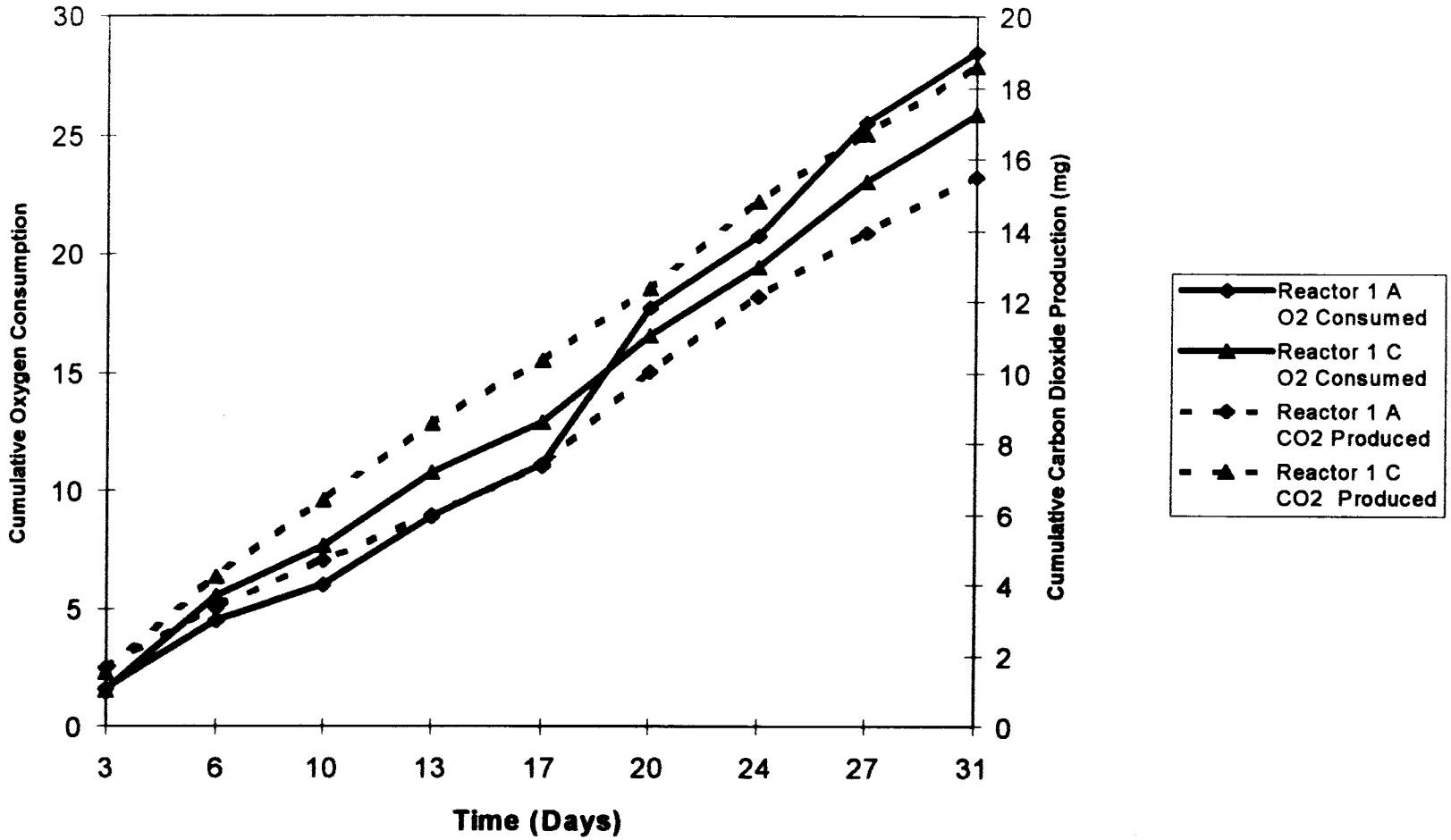

Figure 21. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production in Reactors Containing Contaminated Soil During Experiment #2.

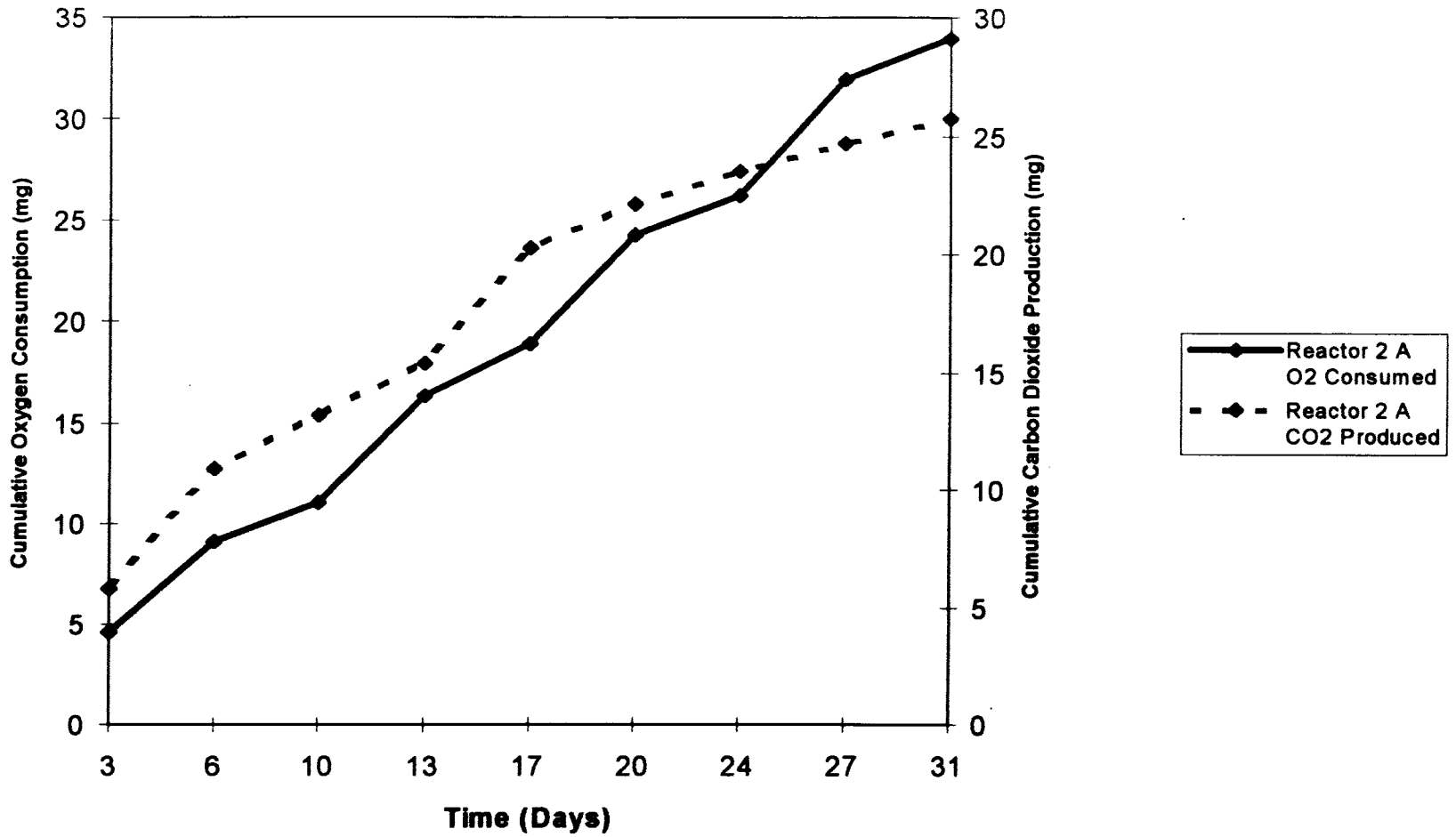

Figure 22. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production in Reactors Containing Uncontaminated Soil with Microbial Amendment and JP-4 Addition During Experiment #2.

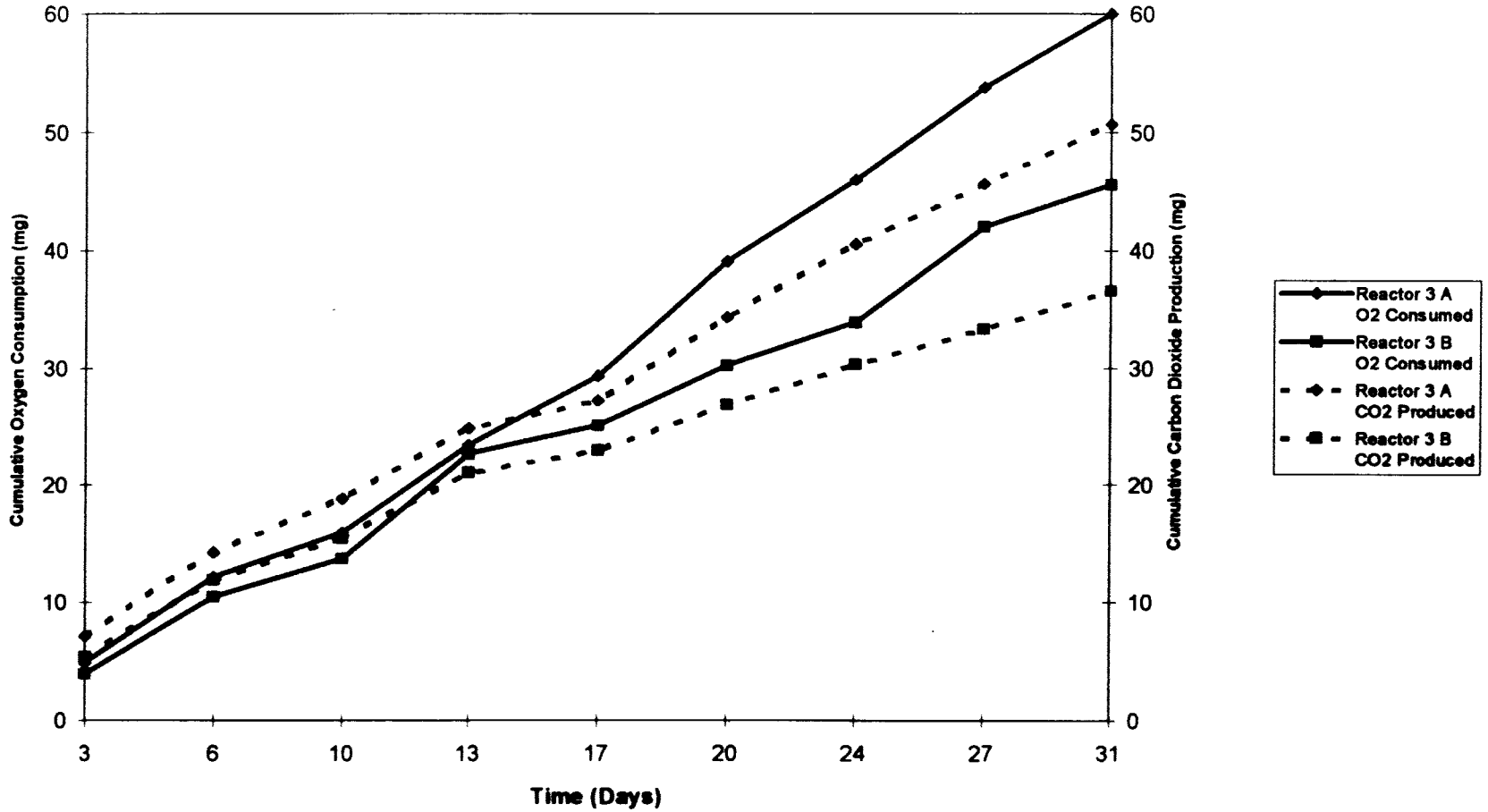

Figure 23. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production (mg) in Reactors Containing Contaminated Soil with the Microbial Amendment During Experiment #2.

Figure 24 shows the average cumulative oxygen consumed in each of the five conditions in Experiment #2. Control 2, the contaminated soil with sterilized amendment, had the highest cumulative oxygen consumption at 56.57 mg. The contaminated soil with the microbial amendment added had the second highest amount of oxygen consumed, the total being 52.74 mg. Reactor 1 and Control 1 had approximately the same amount of oxygen consumed, 27.19 mg and 25.67 mg, respectively. Reactor 1 contained only the contaminated soil and Control 1 contained the uncontaminated soil without any microbial amendment. The uncontaminated soil with the JP-4 jet fuel and the microbial amendment addition had a total of 33.97 mg of oxygen consumed.

5.3.2 Petroleum Hydrocarbons in Gas Samples

The cumulative TPH in gas samples for Control 1 are shown in Figure 25. Control 1 A contained the highest cumulative TPH among the three columns with uncontaminated soil with a total of 2.59 μg after 31 days. Control 1 B and Control 1 C had similar cumulative TPH at 1.75 μg and 1.78 μg, respectively. Large amounts of TPH were not expected in this condition because the soil was uncontaminated.

Figure 26 compares the cumulative TPH of the columns with contaminated soil with sterilized amendment. Control 2 B contained the highest cumulative TPH at 41.33 μg. Control 2 C had a higher amount of TPH than Control 2 A until sampling on day 17. After day 17, Control 2 C added approximately 1 μg of TPH at each sampling and Control 2 A added 2.5 μg at each sampling. The cumulative TPH for Control 2 A was 34.68 μg and the cumulative TPH for Control 2 C was 30.57 μg. The TPH values from the reactors containing the contaminated soil were expected to be higher than those from the reactors containing the uncontaminated soil.

The Reactor 1 A and Reactor 1 C cumulative TPH data are graphed in Figure 27. Reactor 1 C had a higher cumulative TPH value than Reactor 1 A. Reactor 1 C had a cumulative TPH value of 177.15 μg, and Reactor 1 A had 106.96 μg. Reactor 1 contained the contaminated soil without any amendment addition. The TPH values should be comparable to the values for contaminated soil with sterilized amendment, Control 2, because the soils for the two conditions came from the same batch of homogenized soil. However, the TPH values were much higher in the columns with contaminated soil, possibly due to adsorption to the bags during further homogenization.

The cumulative TPH data for uncontaminated soil with JP-4 and microbial amendment addition are exhibited in Figure 28. The Reactor 2 A cumulative TPH gradually rose during the experiment, showing that the TPH levels were slightly decreasing. The cumulative TPH for Reactor 2 A was 15.96 μg. Reactor 2 B

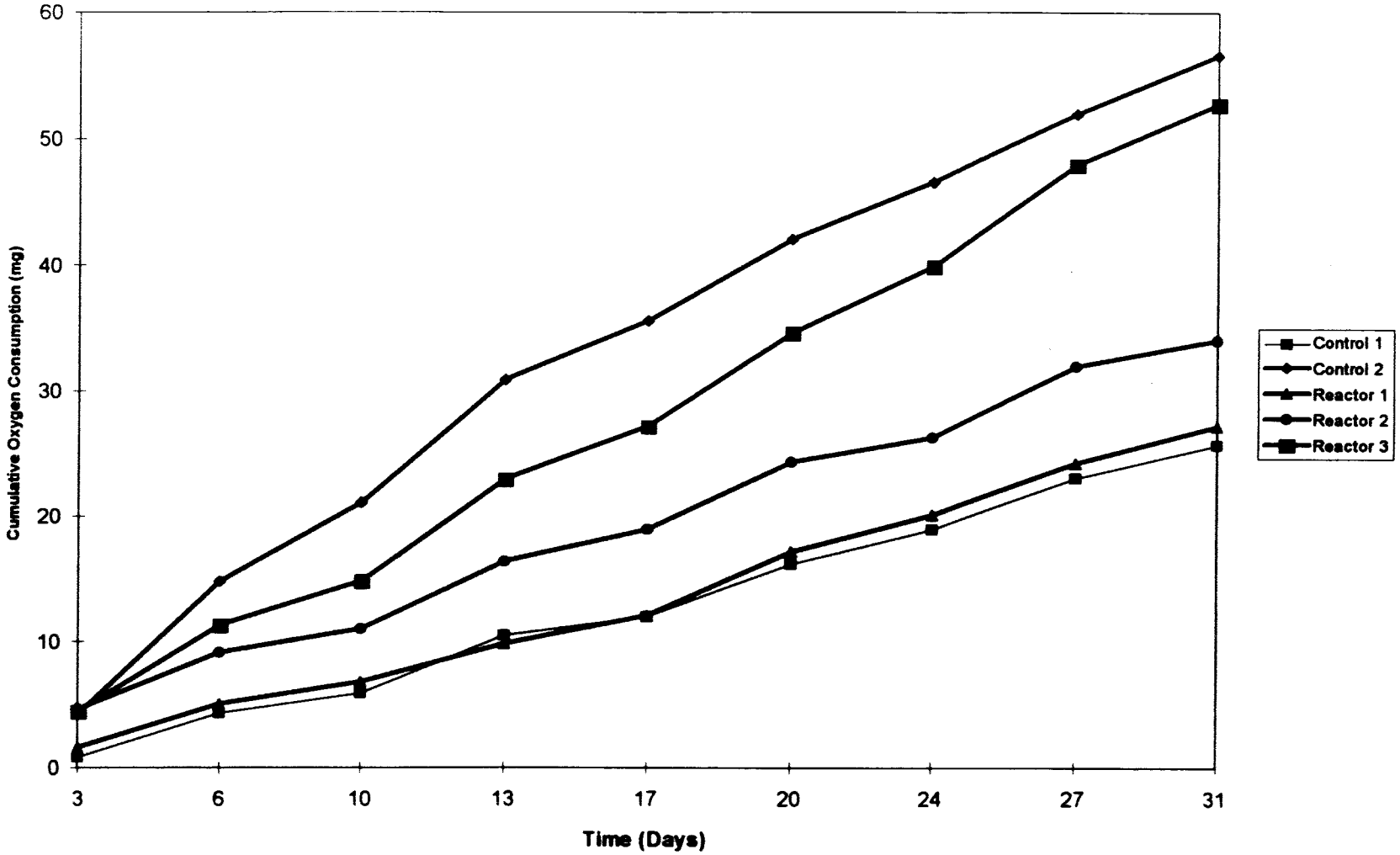

Figure 24. Average Cumulative Oxygen Utilization in Reactors Containing Soils Under Five Experimental Conditions During Experiment #2.

had no increase in cumulative TPH value. The TPH stayed at 4.68 μg throughout the duration of the experiment. The volatilization of the fresh JP-4 accounts for the TPH values being lower for Reactor 2 than for Control 2 and Reactor 1.

Figure 29 shows the cumulative TPH for the contaminated soil with the microbial amendment. Reactor 3 A contained two and a half times more cumulative TPH than Reactor 3 B. The cumulative TPH for Reactor 3 A was 297.61 μg and for Reactor 3 B the cumulative TPH was 104.90 μg. Reactor 3 A TPH was initially three and a half times greater than that of Reactor 3 B and continued to be greater than Reactor 3 B throughout the experiment.

5.3.3 Petroleum Hydrocarbons in Soil Samples

Table 14 depicts the initial and final TPH values for the two controls and three experimental conditions in Experiment 2. A negative average change indicates a final mass that is less than the initial petroleum hydrocarbon mass. A positive average change indicates a final mass that is greater than the initial mass of TPH. The results in Table 14 indicate a decrease in TPH after incubation for each of the experimental conditions and the contaminated control. The removal of TPH was greatest in the experimental condition with added JP-4 and amendment. Approximately 95% of the TPH was removed by the end of the incubation period. The second greatest removal occurred in the experimental condition with sterilized amendment addition, with an average change of approximately 68%. The experimental condition with amendment addition resulted in a TPH removal of approximately 63%, while the contaminated control resulted in a TPH removal of approximately 58%. The uncontaminated control resulted in the only increase in TPH over the 30-day incubation. However, this was most likely due to error associated with the low TPH values encountered in each reactor for this condition.

Initial and final masses of each of the 20 specific petroleum hydrocarbon compounds for the five experimental conditions are presented in Figures 30 through 34. The results depicted in Figure 30 indicate that five of the compounds were determined to have masses less than 2 μg in the uncontaminated soil (Control 1). The final values for these compounds were not detected.

Figure 31 presents the results for the masses of specific compounds detected in the contaminated soil with sterilized amendment control. Twelve initial values were reported, ranging from toluene to *n*-tetradecane. The greatest value reported for this condition was *n*-tetradecane with a reported value of 111 μg. Approximately 62% of this compound was removed by the end of the incubation period.

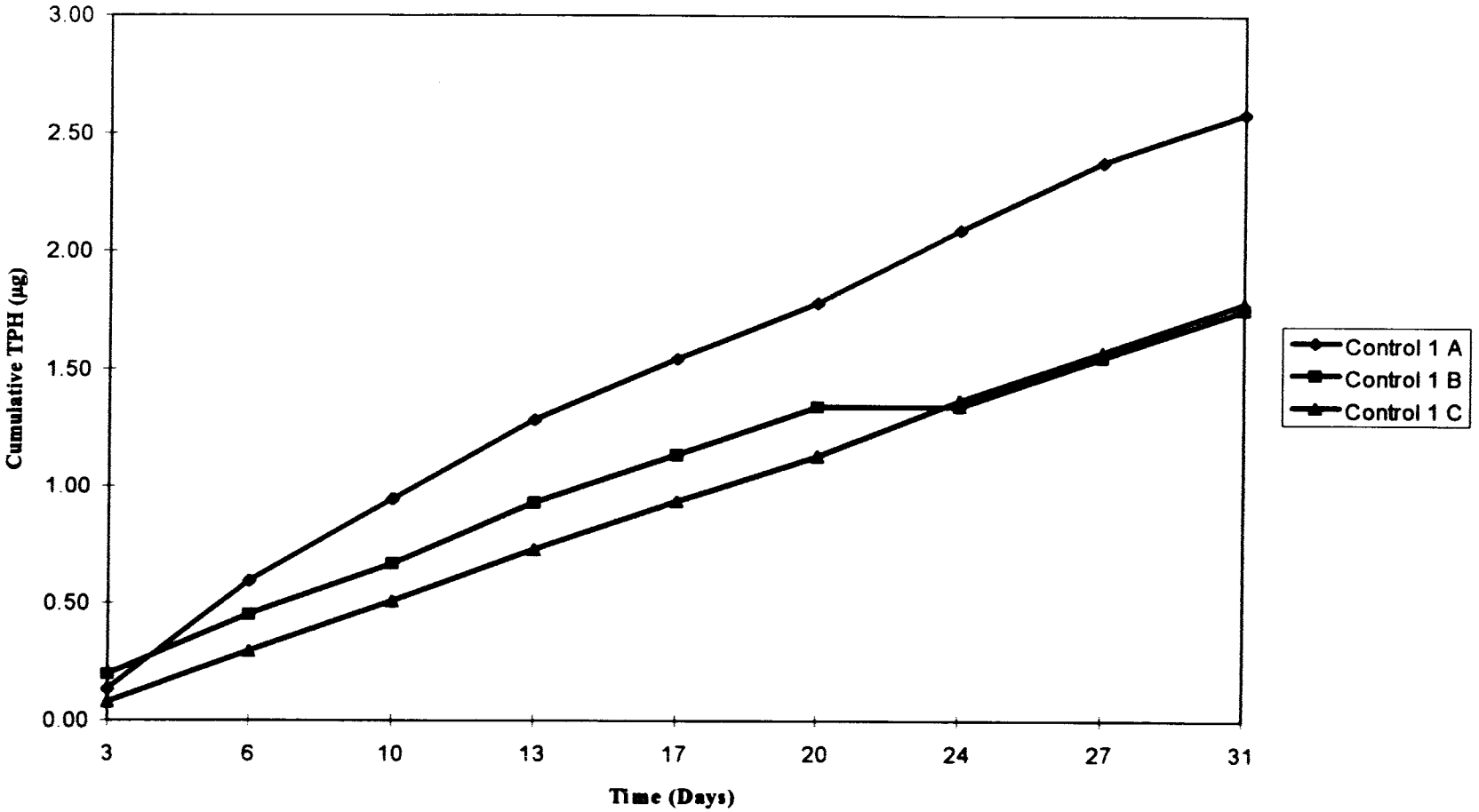

Figure 25. Cumulative TPH (µg) Removed from Reactors Containing Uncontaminated Soil During Atmosphere Exchanging In Experiment #2.

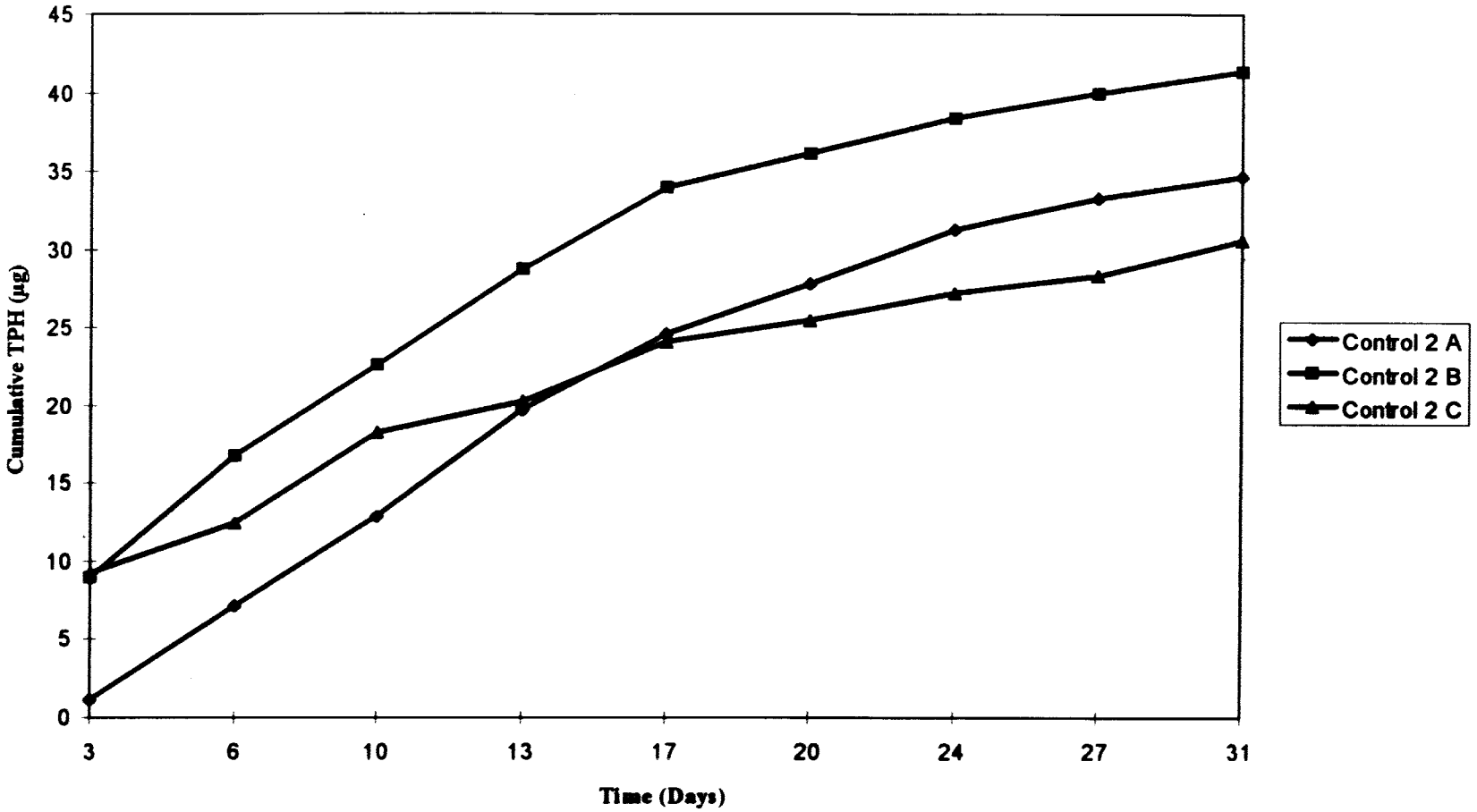

Figure 26. Cumulative TPH Removed from Reactors Containing Contaminated Soil with Sterilized Amendment During Atmosphere Exchanging in Experiment #2.

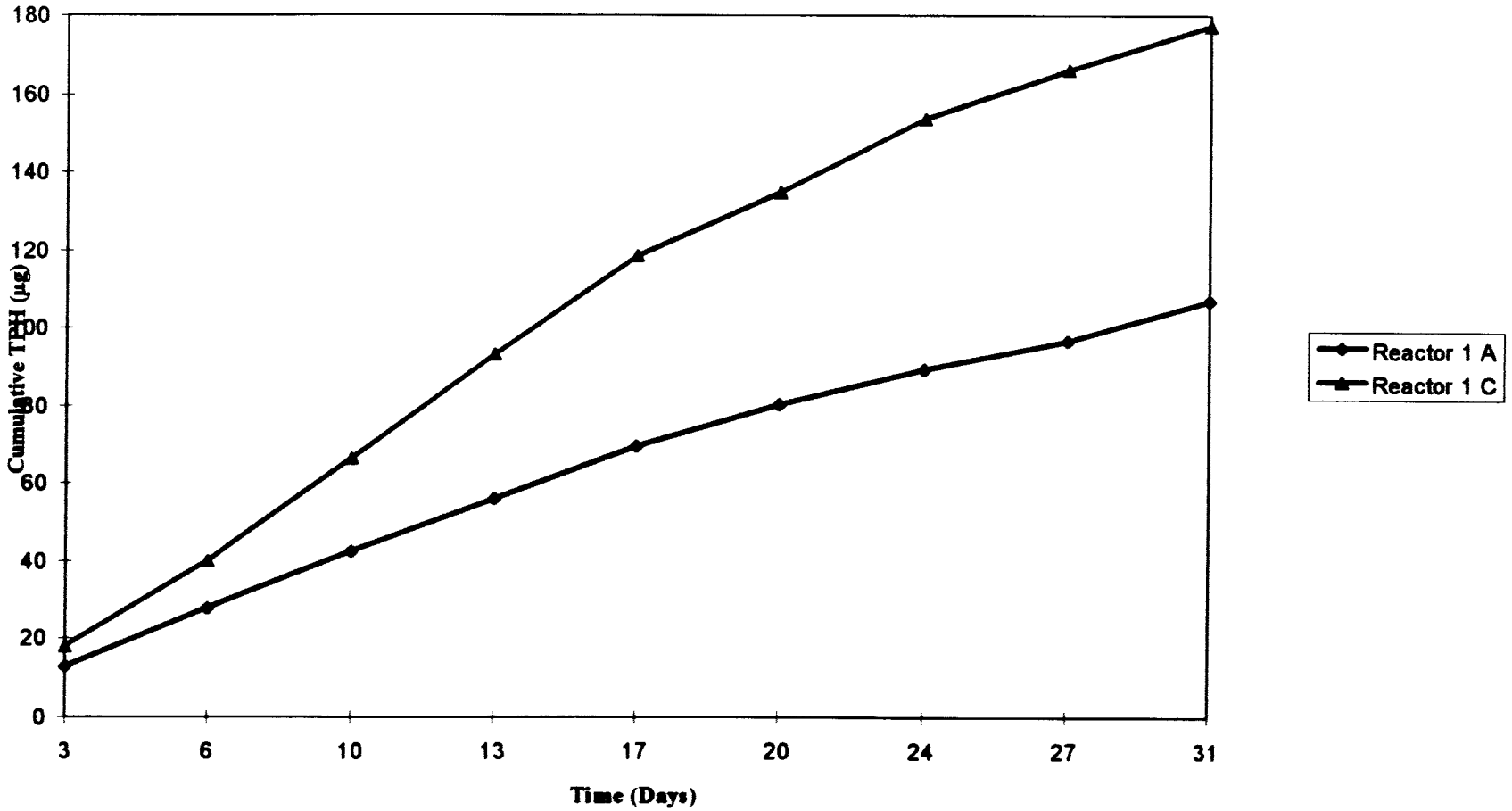

Figure 27. Cumulative TPH (µg) Removed from Reactors Containing Contaminated Soil During Atmosphere Exchanging in Experiment #2.

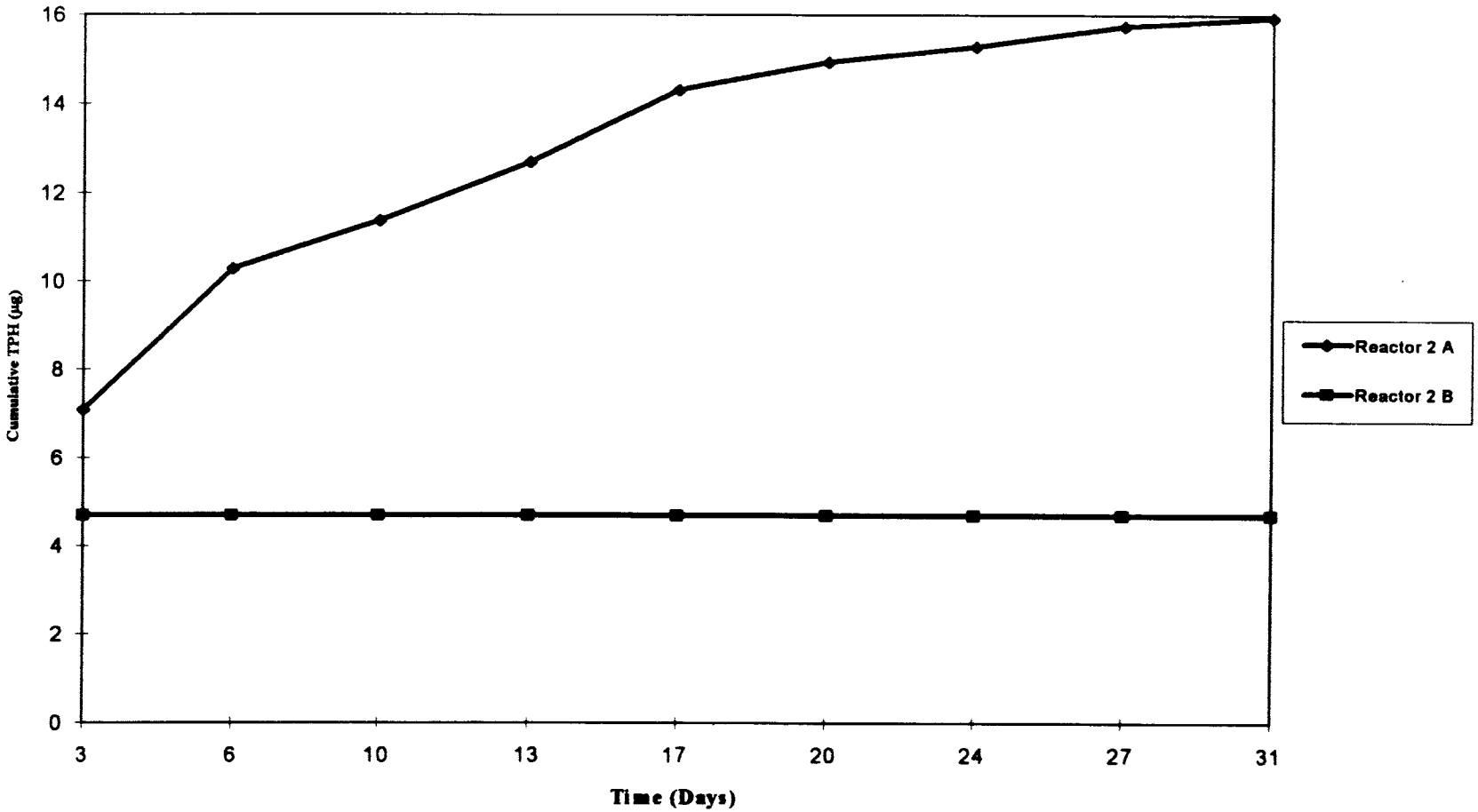

Figure 28. Cumulative Mass of TPH (µg) Removed from Reactors Containing Uncontaminated Soil with JP-4 and Microbial Amendment During Atmosphere Exchanging in Experiment #2.

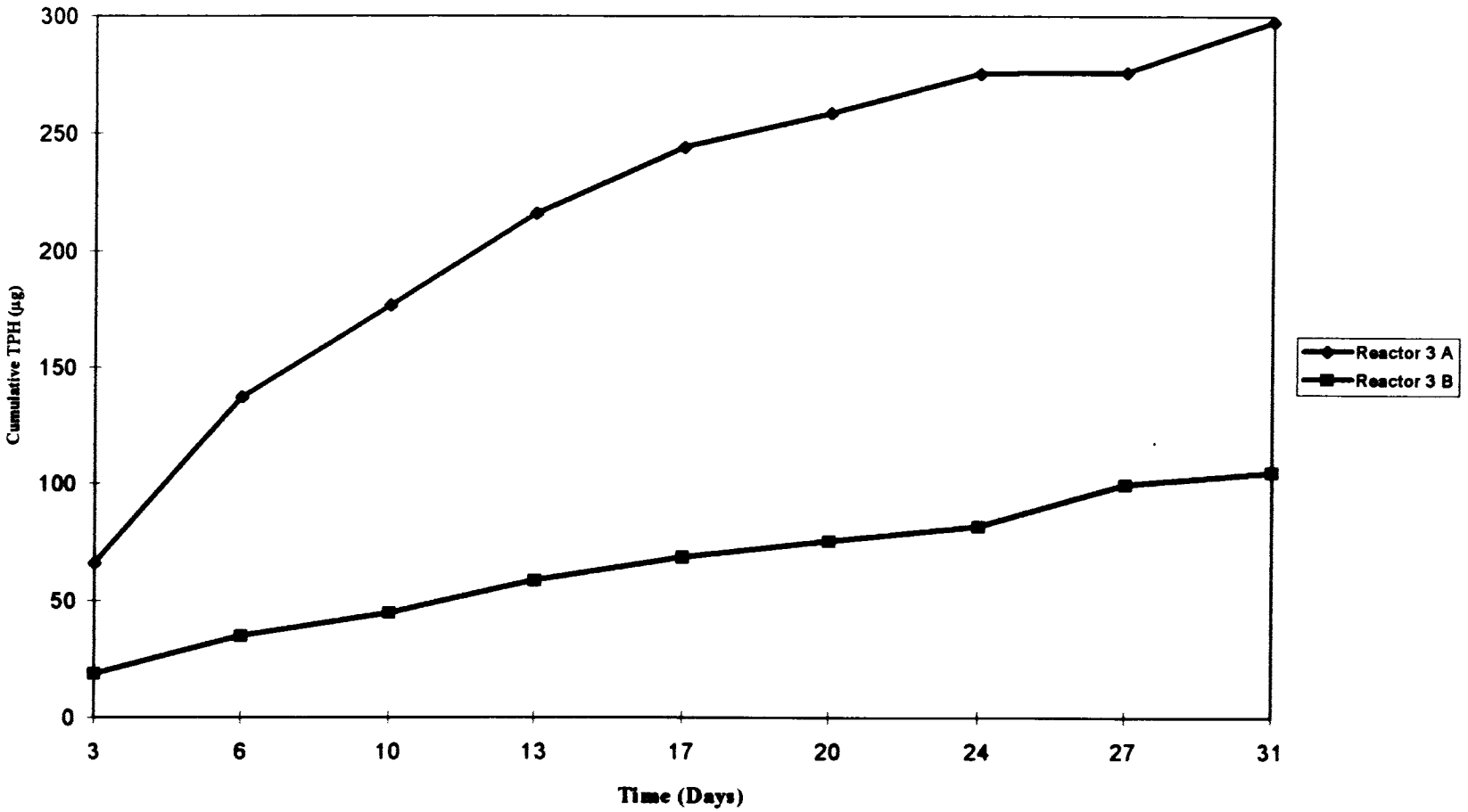

Figure 29. Cumulative Mass of TPH (µg) Removed from Reactors Containing Contaminated Soil with Microbial Amendment During Atmosphere Exchanging In Experiment #2.

The initial and final masses for the experimental condition containing only contaminated soil are shown in Figure 31. The amounts of *n*-dodecane and *n*-tridecane resulted in the greatest initial masses for this condition, with values of 455 µg and 265 µg, respectively. Incubation resulted in approximately 78% removal of *n*-dodecane and approximately 30% of *n*-tridecane.

Figure 33 presents the initial and final masses for the experimental condition with added JP-4 and amendment. Of the 15 initial values were reported for this condition, all masses were extremely low. The greatest initial value reported was only 20 µg for *n*-decane. The low values associated with this condition were a result of the inability to spike uncontaminated soil with JP-4 jet fuel at representative concentrations. To achieve a TPH concentration of approximately 162 mg/kg in 500 g of wet soil, it was necessary to add a 93-µL aliquot of JP-4 jet fuel. It was impossible to obtain a homogeneous sample when working with such low concentrations. The inability to create a representative sample and the fact that this condition stood alone, with nothing to be compared to, resulted in the decision to dismiss this condition in the third experiment.

The initial and final masses for petroleum hydrocarbon compounds in the experimental condition with amendment are presented in Figure 34, which shows the 13 compounds reported for this condition. The greatest masses were reported for *n*-dodecane and *n*-tridecane, with values of 344 µg and 265 µg, respectively. By the end of the incubation, approximately 64% of the *n*-dodecane and approximately 69% of the *n*-tridecane was removed.

5.3.4 Soil Moisture

The resulting soil moisture contents for initial and final reactors in Experiment #2 are presented in Table 13. The negative percent average change values presented in this table signify a loss of water from the reactor system. A positive value indicates a moisture gain inside the reactor. A loss of moisture occurred in all experimental conditions, except for the uncontaminated control. There was a significant moisture decrease in two of these four experimental conditions, i.e., the contaminated soil with amendment condition, and the contaminated soil with added JP-4 and amendment condition. These two conditions exhibited a greater loss of moisture during the second experimentation than in the first. The glass wool that was added in the design setup for Experiment #2 did not seem to be effective in controlling moisture loss.

The uncontaminated control condition was the only condition out of the five that resulted in a moisture increase. The increase in moisture occurred because of a defective water seal in column reactors A and C. The leak occurred at the place where the inner column and the outer water jacket were separated by a rubber o-ring. Leakage became a common problem during Experiment #2. Of the 15 column reactors, 4

Table 13. Results of Soil Moisture Analysis (%) Conducted on Soil Samples During Experiment #2.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	19.39	20.31	15.74	23.23	
2	18.81	21.74	15.67	24.55	
3	18.89	21.38	16.06	22.31	
Average	19.03	21.15	15.82	23.36	5.66
Contaminated Soil with No Amendment					
1	19.55	15.88	NS	16.58	
2	19.85	17.00	NS	16.83	
3	20.19	17.21	NS	16.41	
Average	19.86	16.70	0.00	16.60	-16.18
Contaminated Soil with Sterilized Amendment Addition					
1	20.02	6.88	13.83	25.88	
2	20.12	6.42	12.61	25.06	
3	21.19	9.25	14.85	25.09	
Average	20.44	7.52	13.76	25.34	-23.97
Contaminated Soil with Amendment Addition					
1	19.74	7.86	16.10	NS	
2	19.50	10.38	15.83	NS	
3	19.84	10.47	16.24	NS	
Average	19.69	9.57	16.06	0.00	-34.94
Uncontaminated Soil with Amendment and JP-4 Addition					
1	18.91	12.32	NS	NS	
2	19.01	12.86	NS	NS	
3	19.42	14.10	NS	NS	
Average	19.11	13.10	0.00	0.00	-31.48

Table 14. Total Petroleum Hydrocarbon Mass (mg) in Soil Samples.

Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment				
0.027	0.032	0.034	0.039	29.63
Contaminated Soil with No Amendment				
6.76	2.80	NS	2.87	-58.06
Contaminated Soil with Sterilized Amendment Addition				
5.98	0.035	1.65	2.13	-68.39
Contaminated Soil with Amendment Addition				
6.06	2.78	1.66	NS	-63.37
Uncontaminated Soil with Amendment and JP-4 Addition				
0.65	0.032	NS	NS	-95.08

NS - No sample available due to column loss

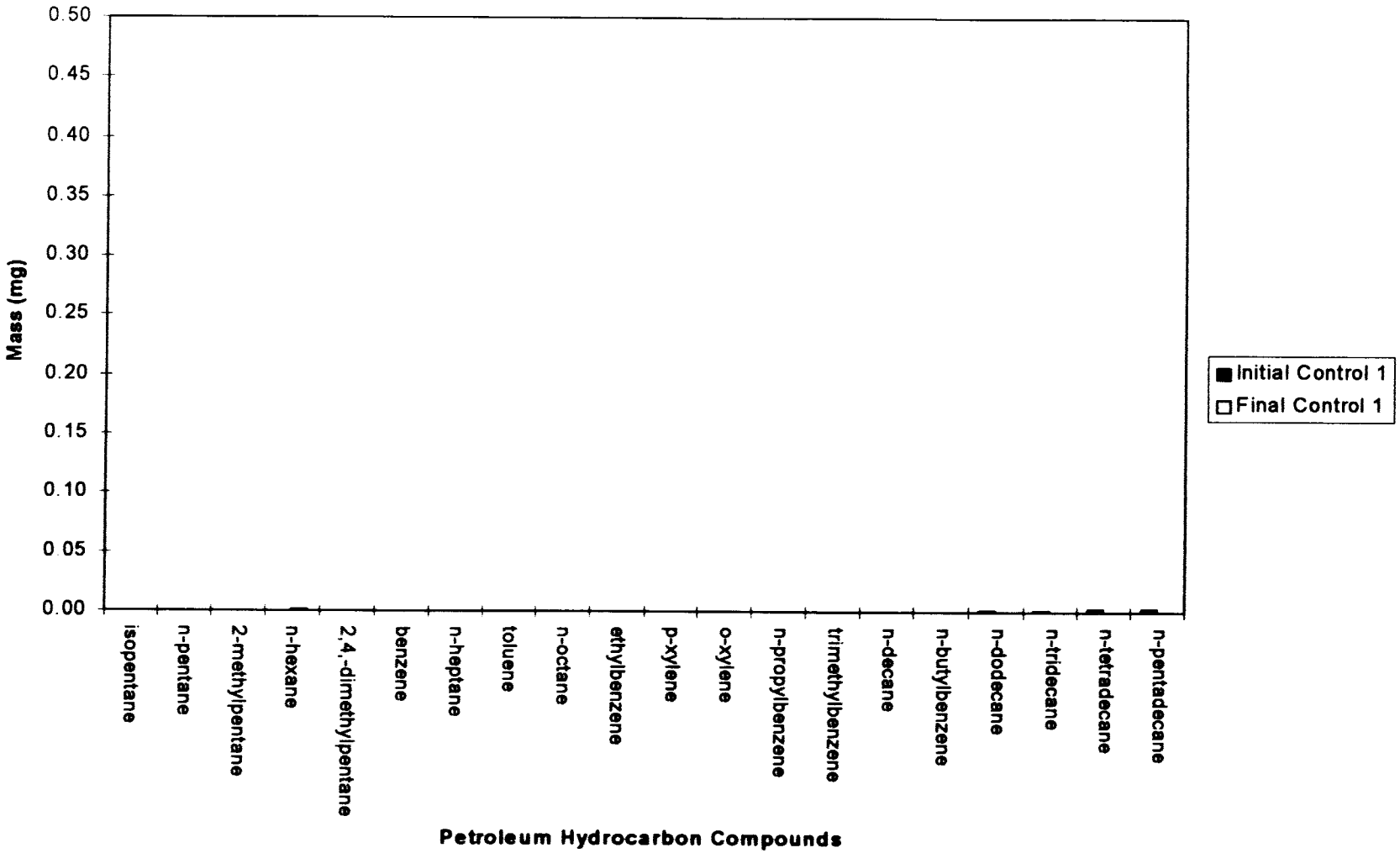

Figure 30. Initial and Average Final Mass of Specific Petroleum Hydrocarbon Compounds (mg) in Reactors Containing Uncontaminated Soil During Experiment #2.

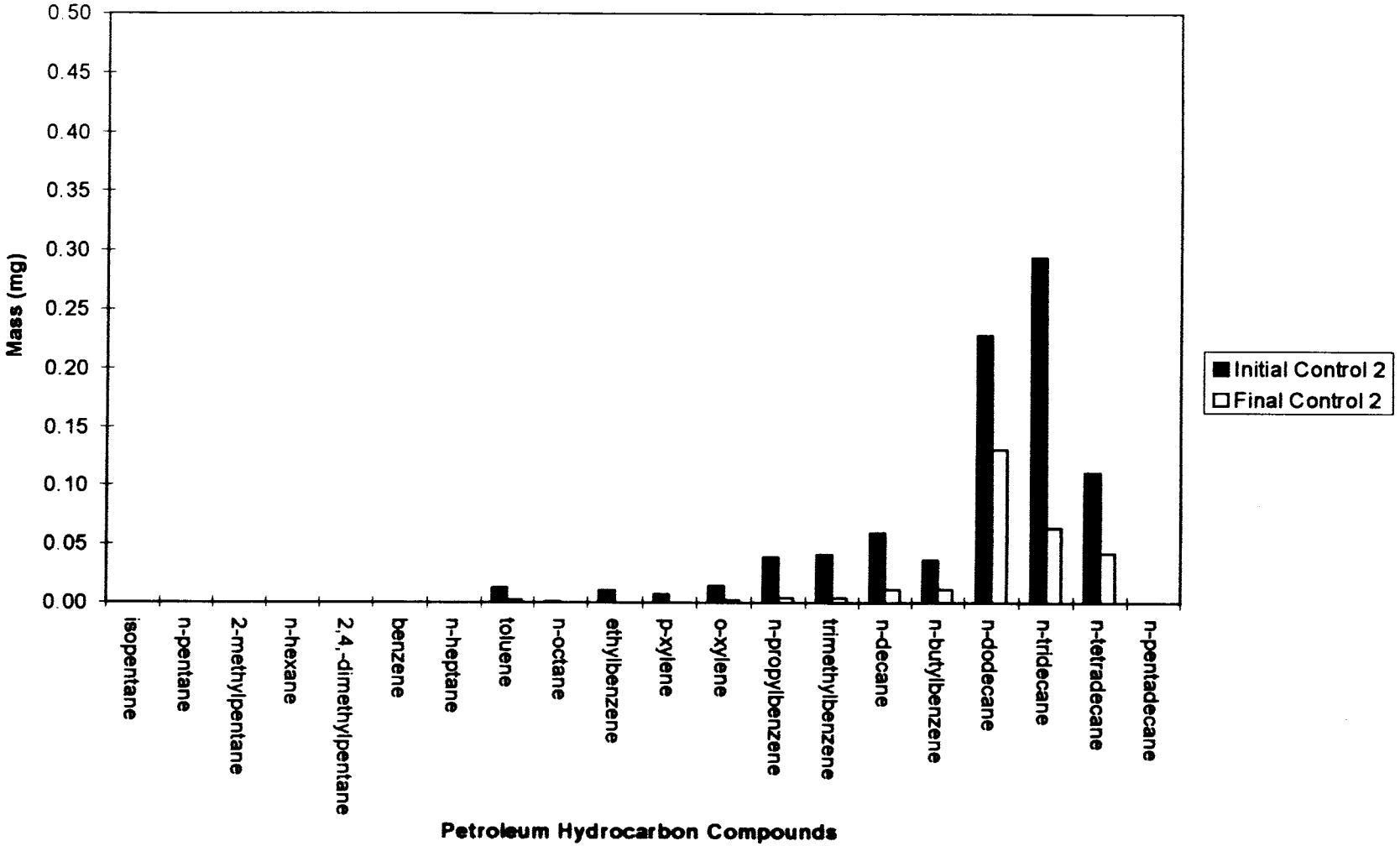

Figure 31. Initial and Average Final Mass of Specific Petroleum Hydrocarbon Compounds (mg) in Reactors Containing Contaminated Soil with Sterilized Amendment During Experiment #2.

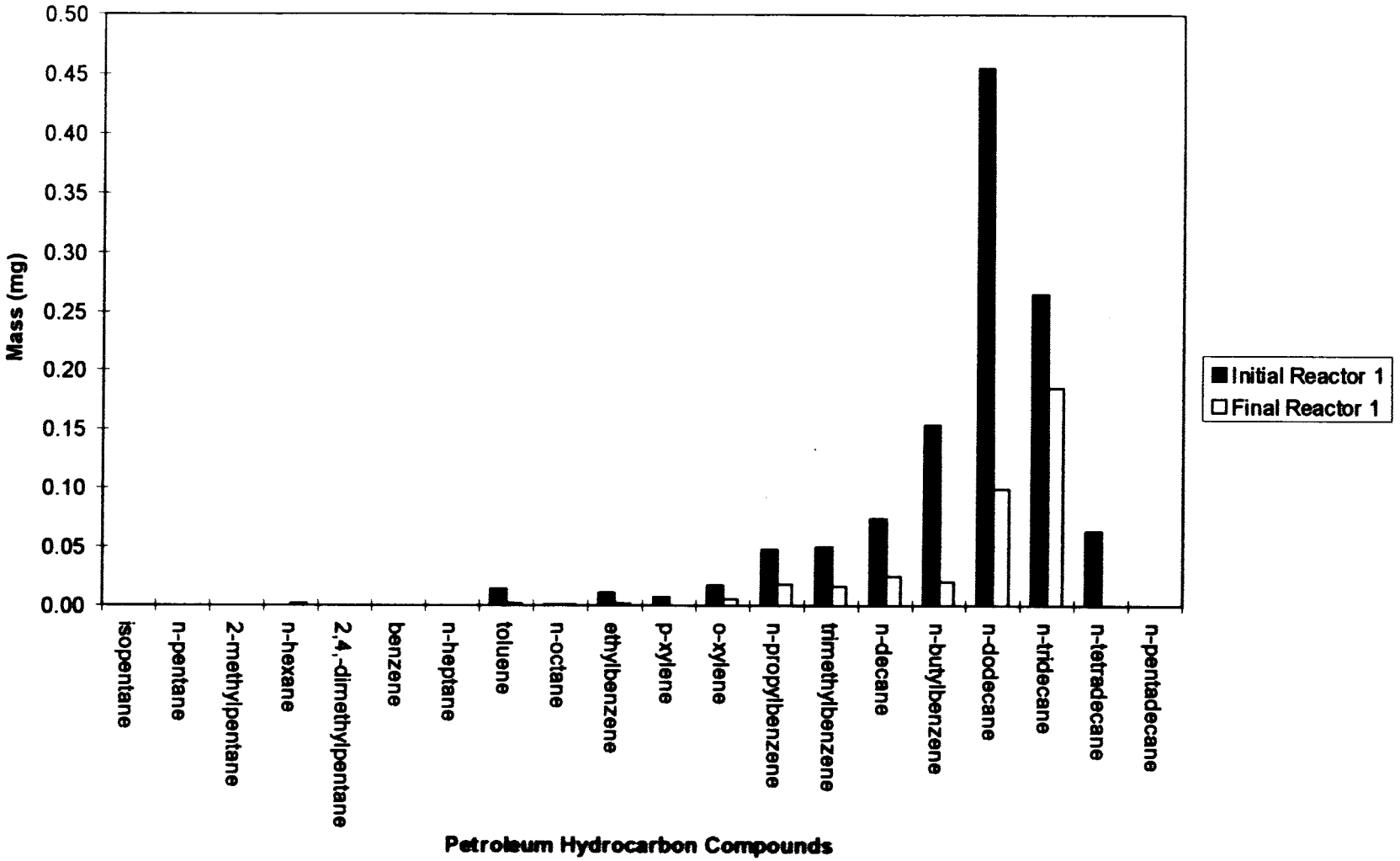

Figure 32. Initial and Average Final Mass of Specific Petroleum Hydrocarbon Compounds (mg) in Reactors Containing Contaminated Soil During Experiment #2.

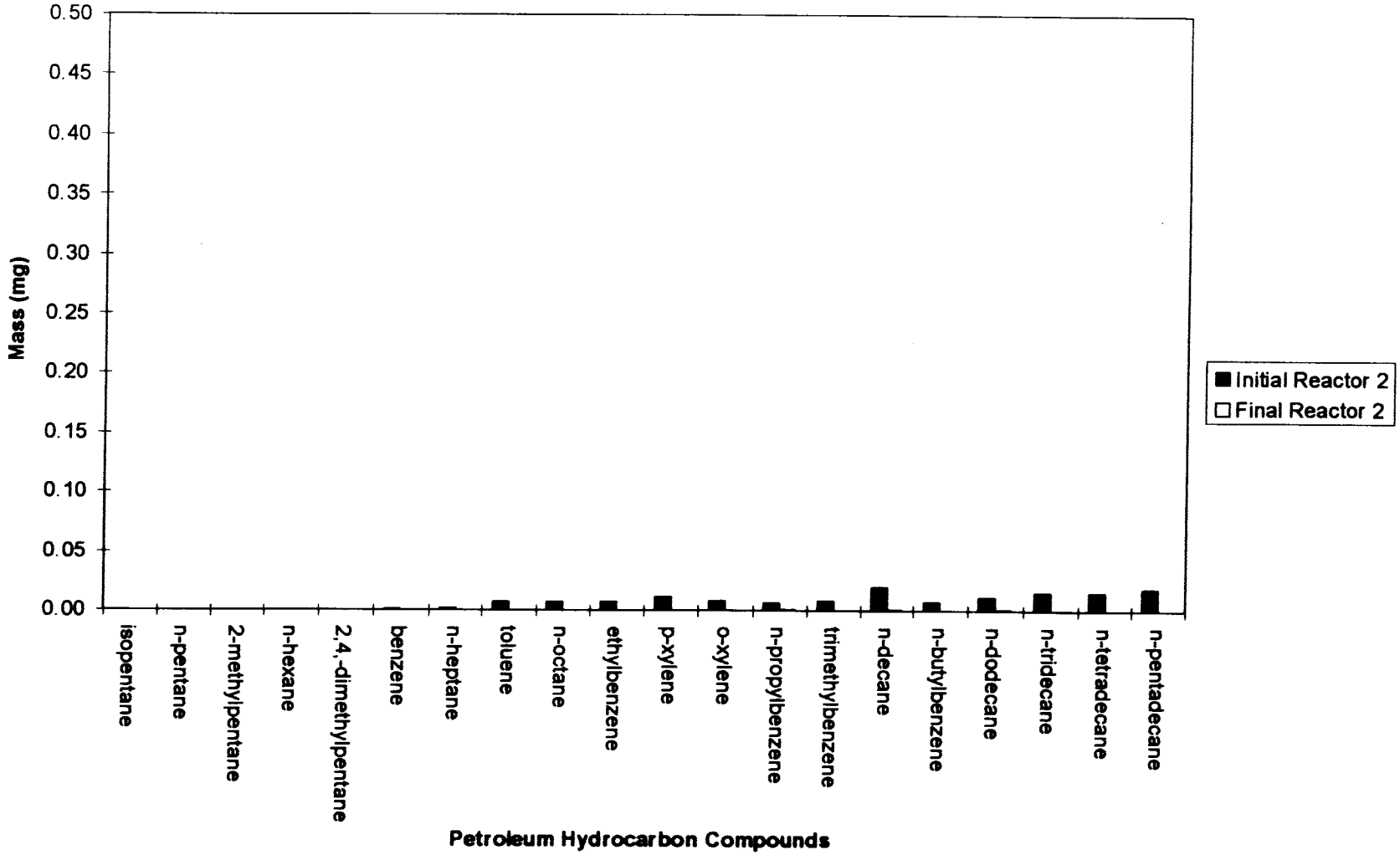

Figure 33. Initial and Average Final Mass of Specific Petroleum Hydrocarbon Compounds (mg) in Reactors Containing Uncontaminated Soil with added JP-4 and Microbial Amendment During Experiment #2.

were lost due to leaks. They were removed from the floor rack and dismantled because of the moisture contamination. The columns that leaked included one from the contaminated soil without amendment condition, one from the contaminated soil with amendment condition, and two from the uncontaminated soil with added JP-4 and amendment condition. Table 13 depicts these reactors as "NS" (no sample).

Even though the o-rings were replaced, leakage still occurred. The o-ring type suggested by the manufacturer did not seal the end of the column properly, even though extreme care was taken during the installation of these o-rings. The water jacket design proved to be an awkward and somewhat troublesome design for this reactor system. Studies were conducted in the next set of experiments to determine an easier way to maintain a constant incubation temperature and a more accessible reactor system for sampling.

5.3.5 Soil pH

The results of the soil pH measurements made before and after the 30-day incubation are presented in Table 15. The initial pH values were approximately 1 unit value less than the initial values reported for Experiment #1 in all of the experimental conditions, except in the uncontaminated soil control. A negative pH change indicated a decrease in pH from the initial measurement made for that condition. This condition occurred in the uncontaminated control and in the uncontaminated soil with JP-4 and amendment addition. The remaining three experimental conditions resulted in a slight pH increase.

5.3.6 Dehydrogenase Activity in Soil Samples

The results for dehydrogenase activity measurements made in Experiment #2 are presented in Table 16. As was the case in Experiment #1, the data from this experiment appeared scattered and no reasonable conclusions could be drawn. The increases and decreases in dehydrogenase activity were not significant. The modified procedure decreased the amount of time spent to conduct the analysis, and also reduced the amount of methanol waste produced during the extraction by 50%. However, these modifications did not appear to enhance the detection of TPF.

The modified dehydrogenase procedure conducted in Experiment #2 confirmed that the low-level detection of TPF in Experiment #1 was not due to cotton absorption effects, because similar values were obtained in this experimental run after the cotton filters were eliminated.

Because there was no significant increase in the final values associated with this experiment, and cotton absorption was ruled out as a probable cause, further investigations were made to address the sensitivity of detection of TPF in Experiment #3.

Table 16. Results of Dehydrogenase Activity Analyses (μg-H/g-dry soil) Conducted on Soil Samples During Experiment #2.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	8.12e+02	1.19e+03	1.18e+03	1.07e+03	
2	7.50e+02	1.05e+03	1.06e+03	7.32e+02	
3	6.87e+02	1.36e+03	7.96e+02	1.51e+03	
Average	7.50e+02	1.20e+03	1.01e+03	1.10e+03	47.44
Contaminated Soil with No Amendment					
1	1.09e+04	1.12e+03	NS	1.05e+03	
2	1.79e+03	1.16e+03	NS	1.22e+03	
3	1.66e+03	1.72e+03	NS	7.42e+03	
Average	4.78e+03	1.33e+03	0.00e+00	3.23e+03	-52.30
Contaminated Soil with Sterilized Amendment Addition					
1	4.00e+03	1.77e+03	1.83e+03	2.54e+03	
2	6.80e+03	1.95e+03	1.42e+03	3.14e+03	
3	4.66e+03	1.57e+03	3.18e+03	1.16e+03	
Average	5.15e+03	1.76e+03	2.14e+03	2.28e+03	-59.98
Contaminated Soil with Amendment Addition					
1	5.17e+03	4.47e+03	2.89e+03	NS	
2	5.82e+03	4.32e+03	2.48e+03	NS	
3	4.11e+03	4.40e+03	3.18e+03	NS	
Average	5.03e+03	4.40e+03	2.85e+03	0.00e+00	-28.01
Uncontaminated Soil with Amendment and JP-4 Addition					
1	1.73e+03	3.83e+03	NS	NS	
2	1.73e+03	2.60e+03	NS	NS	
3	1.84e+03	4.31e+03	NS	NS	
Average	1.77e+03	3.58e+03	0.00e+00	0.00e+00	102.64

NS No sample due to the loss of this reactor because of water interference.

5.3.7 Microbial Enumerations in Soil Samples

The uncontaminated soil microbial count increased tenfold in the 31 days of the experimental run. The contaminated soil and the contaminated soil with sterilized amendment both had an increase in microbial numbers. The uncontaminated soil, the contaminated soil, and the contaminated soil with sterilized amendment were counted from 10^{-2} to 10^{-6}. An initial microbial count was not obtained on the contaminated soil with amendment nor on the uncontaminated soil with amendment and JP-4 addition. These conditions were counted 10^{-6} to 10^{-11} because of the results of direct plate counts performed on inoculum made June 26, 1995. The batch of microbial amendment for the direct count was made 100 times the concentration of the first experiment, making the total cells/mL 9.7×10^{8}. The amendment added in this experiment had the same amount of nutrients as for the first experiment, but 100 times the microbes. By using the direct count data and the moisture content data from the soil samples in this experiment, it was determined that approximately 3.2×10^{7} cells per gram of dry soil was added to uncontaminated soil for the uncontaminated soil plus JP-4 and amendment condition and to contaminated soil for the contaminated soil with amendment condition. The inoculum batch used also was plated onto the basal inorganic medium plus JP-4 to produce an actual count of the microbes that were added. The inoculum also was plated 10^{-6} to 10^{-11} onto the basal inorganic plus JP-4 medium. The inoculum had 1.5×10^{5} colony forming units (CFU)/mL. The inoculum added was 100 times less than the direct cell count on the batch of inoculum made on June 26, 1995. For the final enumerations, the uncontaminated soil with JP-4 and amendment addition and the contaminated soil with amendment addition were plated 10^{-3} to 10^{-9} to ensure that microbial counts could be made.

5.3.8 Nutrient Concentrations in Soil Samples

Nutrient analyses were not included in Experiment #2 for the reasons discussed in Section 5.2.8. Although these analyses were considered critical and are included in the final protocol, it was assumed that nutrient concentrations would be similar to the concentrations obtained for Experiment #1, because the same soils and the inoculum nutrient concentrations were used in Experiments #1 and #2.

5.3.9 Carbon in Soil Samples

The results for the inorganic and organic carbon analyses conducted for Experiment #2 using the UIC method of analysis are presented in Table 17 and Table 18, respectively. The data for both inorganic and organic carbon were once again scattered, as mentioned in Section 4.3.9 for the inorganic and organic carbon analyses conducted in Experiment #1. The values for the replicate samples taken from each reactor indicate extreme variability in some conditions. The inability to reproduce results within replicate samples of

the same flask is indicative of a nonhomogenous sample. As explained previously in Experiment #1, it is believed that this heterogeneity is derived from the inclusion of larger soil particles, such as rocks, roots, and other organic debris, and from the variability in the moisture content from one sample to the next because of the small sample size (approximately 200 mg).

The results for organic carbon analyses using the muffle method in Experiment #2 are presented in Table 19. The resulting values were approximately double the values obtained for organic analyses using the UIC method. The increased sample size resulted in a more representative sample that included various root particles and other organic debris which contributed to the greater organic carbon values. However, reactor replicates still deviated significantly, resulting in skewed values for the average percent change and an inability to draw relative conclusions among the experimental and control conditions. If this variability within the three reactor replicates continues, it may become necessary to increase the number of replicates sampled or to continue to sample until the results fall within a predetermined error range.

5.3.10 Particle Size Distribution

Analyses of soil particle size and percent sand, silt, and clay were not conducted in Experiment #2. These analyses are important and are included in the final protocol. However, it was assumed that these parameters would remain consistent with the results obtained during Experiment #1 as the same batch of soil was used for this experiment.

5.3.11 Cation Exchange Capacity

Cation exchange capacity analyses were not conducted for Experiment #2, as explained previously in Section 4.2.11. The analysis is considered important and is included in the final protocol. However, because the same batch of soil was used in Experiments #1 and #2, it was concluded that repeating the analysis would be unnecessary.

Table 17. Results from Inorganic Carbon Analysis (ppm) Using the UIC Method on Soil Samples During Experiment #2.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	474	335	351	230	
2	529	203	227	247	
3	714	306	203	242	
Average	572	281	260	240	-54.47
Contaminated Soil with No Amendment					
1	460	187	NS	394	
2	411	231	NS	NS	
3	403	532	NS	424	
Average	425	317	0	409	-14.48
Contaminated Soil with Sterilized Amendment Addition					
1	308	198	190	395	
2	424	224	188	361	
3	621	241	264	310	
Average	451	221	214	378	-39.94
Contaminated Soil with Amendment Addition					
1	350	318	221	NS	
2	294	226	317	NS	
3	259	403	NS	NS	
Average	301	315	317	0	4.97
Uncontaminated Soil with Amendment and JP-4 Addition					
1	736	392	NS	NS	
2	614	369	NS	NS	
3	575	274	NS	NS	
Average	641	345	0	0	-46.20

Table 18. Results of Organic Carbon Analysis (mg-C/kg-dry soil) Using the UIC Method on Soil Samples During Experiment #2.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	7,319	6,946	8,068	8,817	
2	12,154	7,907	7,799	7,242	
3	7,296	7,743	7,667	7,639	
Average	8,923	7,532	7,845	7,899	-13.05
Contaminated Soil with No Amendment					
1	7,321	8,563	NS	6,815	
2	6,976	8,397	NS	6,714	
3	7,034	6,726	NS	6,896	
Average	7,110	7,896	0	6,808	3.40
Contaminated Soil with Sterilized Amendment Addition					
1	7,044	6,261	8,464	8,252	
2	7,540	6,336	7,018	9,405	
3	7,791	5,973	6,602	8,648	
Average	7,459	6,190	7,362	8,769	0.25
Contaminated Soil with Amendment Addition					
1	9,310	7,989	8,747	NS	
2	7,309	6,921	7,172	NS	
3	7,127	6,938	6,875	NS	
Average	7,916	7,283	7,598	0	-6.00
Uncontaminated Soil with Amendment and JP-4 Addition					
1	8,149	8,421	NS	NS	
2	8,013	8,153	NS	NS	
3	8,471	10,905	NS	NS	
Average	8,211	9,160	0	0	11.55

NS No sample due to loss of this reactor because of water interference.

Table 19. Results of Organic Carbon (Matter) Analysis (mg-C/kg-dry soil) Using the Muffle Furnace Method for Soil Samples During Experiment #2.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Uncontaminated Soil with No Amendment					
1	16,273	16,462	16,145	16,227	
2	15,422	NS[2]	13,340	15,323	
3	15,667	17,845	7,078	16,149	
Average	15,787	17,154	12,188	15,900	62.19
Contaminated Soil with No Amendment					
1	12,992	46,741	NS[1]	30,251	
2	15,296	14,011	NS[1]	4,881	
3	14,594	13,709	NS[1]	12,081	
Average	14,294	24,820	0	15,738	41.87
Contaminated Soil with Sterilized Amendment Addition					
1	14,241	13,402	13,164	NS[2]	
2	13,880	16,298	NS[2]	13,002	
3	26,202	NS[2]	NS[2]	13,214	
Average	18,108	13,402	13,164	13,108	-26.97
Contaminated Soil with Amendment Addition					
1	14,135	58,381	13,659	NS[1]	
2	13,770	14,289	13,300	NS[1]	
3	13,890	13,527	18,749	NS[1]	
Average	13,932	28,732	15,236	0	57.80
Uncontaminated Soil with Amendment and JP-4 Addition					
1	16,731	15,200	NS[1]	NS[1]	
2	16,289	NS[2]	NS[1]	NS[1]	
3	17,576	14,537	NS[1]	NS[1]	
Average	16,865	14,869	0	0	-11.84

NS[1] No sample due to the loss of this reactor because of water interference.
NS[2] No sample due to sample handling error.

6.0 EXPERIMENT #3

The third experimental run was the final run in the development of the protocol. Certain experimental and analytical methods were further refined and the experiment included both column and biometer flask reactor configurations. Three experimental conditions were examined, with the uncontaminated soil control and the added JP-4 conditions being omitted. These conditions did not provide any meaningful data and their inclusion would have resulted in the generation of too much waste and would have required too much staff time. Thus, these two conditions were dropped so that the effort could focus on the two reactor configurations.

The inoculum was modified so that the bacterial inoculation rate was the same as in Experiment #2 and the nutrient addition was increased tenfold. Because the nutrient addition was modified, soil samples were analyzed for nutrient concentrations and physical properties (pH, CEC, particle size distribution, etc.). The following sections contain descriptions of the experimental and analytical methods employed during this experimental run.

6.1 Experimental Methods

The experimental methods used during this experimental run included modifications to the reactor design, the method for maintaining temperature, and the method for exchanging atmospheric gases. The following sections contain descriptions of the modified methods for accomplishing these tasks.

6.1.1 Soil Processing

The uncontaminated soil control condition was not included in this experiment and was not prepared. Preparation of the batches of contaminated soil followed the procedure described in Section 4.1.1. Single batches of contaminated soil were prepared for experimental conditions #2, #3, and #4, and each batch of the homogenized soil was used for both the column and the biometer flask reactors. This allowed for better reproducibility of experimental results.

6.1.2 Reactor Design and Setup

The reactor design was modified for Experiment #3 due to the leakage problem encountered with the water circulation system during Experiment #2. Nine of the column-type reactors were configured without a water-jacket assembly. Each column was filled with 87 g of the wet soil prepared as explained in Section 4.1.2. The nine reactors represented triplicate sets of each of the three experimental conditions being tested

in Experiment #3. Small pieces of glass wool were placed at each of the end caps to separate the soil/Teflon™-cap interface. After the soil was loaded into the reactors, the endcaps were fastened and the columns were placed on their sides in a bench-top model Thermolyne® incubator maintained at 25°C.

In addition to the column-type reactors, nine biometer flask reactors were included in this experiment. As with the column reactors, the biometer flask reactors were set up by adding 87 g of wet soil as described in Section 4.1.2. The nine reactors represented triplicate sets of each of the three experimental test conditions. A schematic diagram of the biometer flask assembly used in Experiment #3 is shown in Figure 35. After the soil was loaded into the reactor, the opening of the biometer flask was sealed with a rubber stopper and each of the two valves were closed. The biometer flasks reactors were placed in an upright position within the same bench-top model incubator as the column reactors.

The 18 reactors were incubated for 30 days at 25°C. The incubator temperature was monitored daily to ensure the proper incubation temperature. The reactors were removed from the incubator only to conduct routine atmospheric exchanges and were promptly placed back into the incubator when the exchange was completed. After the 30-day incubation period, the reactors were removed from the incubator and harvested for analyses of the incubated soils.

6.1.3 Reactor Operation and Monitoring

6.1.3.1 Maintaining Temperature. The biometer flasks and columns were placed in the same incubator to maintain a constant temperature at 25°C. The columns were laid on their sides and placed on the shelf in the incubator. The incubator was monitored regularly to ensure that the temperature remained constant.

6.1.3.2 Atmosphere Exchanges. Atmospheric gases were exchanged every 3 days in both the column and the biometer flask reactors. The atmospheres in the columns were exchanged according to the same procedure used during the previous experiments as described in Section 4.1.3.2.

The apparatus used to exchange the atmospheres in the biometer flasks apparatus was the same as used for the columns. However, the method was different because of the different valving configuration. To exchange the gases in the biometer flasks, the desiccator influent line was attached to a three-way valve at the top of the flask sidearm. A 22-gauge syringe needle was fitted with a two-way stopcock and was inserted into the Neoprene™ rubber stopper placed in the Ascarite™ chamber on the top of biometer flask.

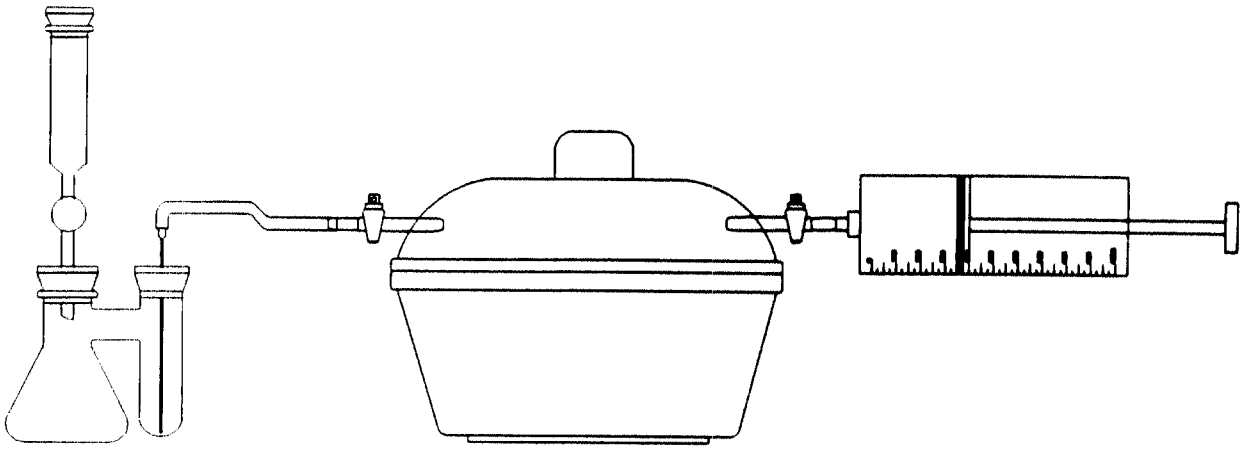

Figure 35. Schematic of the Apparatus Used to Exchange Atmospheres in the Biometer Flask Reactors During Experiment #3.

To exchange a measured volume of gas, a Tedlar™ gas sampling bag was filled with 100 cm^3 of clean lab air and attached to the stopcock on the needle in the Neoprene™ rubber stopper. A vacuum was established in the vacuum desiccator by pulling approximately 800 cm^3 of air using a 1-liter syringe. To facilitate gas exchange, the valves on the biometer flask were opened in the following order: (1) valve one of the desiccator, (2) the three way valve on the arm of the biometer flask, (3) the valve on the Ascarite™ chamber, (4) the two-way stopcock on the needle, and finally (5) the valve on the Tedlar™ bag connected to the stopcock.

After all of the 100 cm^3 of air was pulled from the Tedlar™ bag, valves 3, 4, and 5 were closed. Valves 1 and 2 remained open, until the desiccator and biometer flask equilibrated at atmospheric pressure. These valves were then closed and the Tedlar™ bag containing the gas sample was removed. The three-way valves on the biometer flasks resulted in operational problems and were replaced with two-way valves after the fourth sampling event.

During the fifth sampling event, the biometer flasks were first directly sampled, then sampled as described above. Direct sampling was accomplished by inserting a 6-inch 18-gauge stainless steel needle with a Luer lock fitting and a two-way valve through the Neoprene™ rubber stopper at the base of the Ascarite™ chamber. The needle was positioned so that the tip was approximately 1 cm above the surface of the soil. A 50-mL gastight syringe was connected to the two-way valve and a 20-mL gas sample was drawn from the biometer flask. Next, 15 mL of the gas sample was injected into the GC to measure oxygen and carbon dioxide concentrations. After the 20-mL sample was collected, the biometer flasks were sampled as before by exchanging 100 mL of clean lab air.

On the seventh sampling series, a new method was developed and used to exchange the atmospheres in both the columns and biometer flasks. The new method was designed to allow for better flowrate control to provide for better flushing and collection of a more representative sample. In the new method, the vacuum desiccator assembly was eliminated. For the columns, 100 mL of clean lab air was pushed through the column in an upward direction using the 1-liter syringe. The effluent air was collected in a helium-flushed Tedlar™ sample bag attached to the valve at the top of the column.

For the biometer flask, the helium-flushed Tedlar™ sample bag was attached to the two-way valve on the side arm of the biometer flask. The use of the Tedlar™ bag containing 100 mL of clean lab air was eliminated. In its place, 100 mL of clean lab air was pushed through the flask using the 1-liter syringe. The syringe was fitted with a piece of Tygon™ tubing and attached to the two-way valve on the 6-inch stainless steel needle. The 100 mL of clean lab air was injected into the flask at a slow flowrate (approximately 100 mL/60 seconds)

For the eighth and final sampling series, the direct extraction procedure was modified to include withdrawal of a 20-mL sample using a 25-mL syringe. Prior to analysis, the syringe was "burped" to equilibrate the syringe chamber to atmospheric pressure to avoid potential dilution problems. Once equilibrated, a 10-mL sample volume was injected into the GC.

6.1.3.3 Monitoring Respiration. The oxygen, carbon dioxide, and petroleum hydrocarbon concentrations in exchanged gas were measured according to the same methods used in the first experiment as described in Section 4.1.3.3.

6.1.4 Reactor Harvesting

After the 30-day incubation period, the reactors were harvested and the soils were subjected to the suite of analyses described in Section 6.2. Individual reactors were removed from the incubator during harvesting with the nine column reactors being harvested first, followed by the nine biometer flask reactors. The column reactors were harvested according to the procedure used during Experiments #1 and #2 as described in Section 4.1.4.

The biometer flask reactors were harvested individually, in the same order of prioritization as in Experiment #1 and #2. All reactors were harvested identically for reasons previously explained. The rubber stopper of each flask was removed and aliquots of soil were collected and placed in their respective containers. Between each sampling event, the rubber stopper was replaced to minimize any loss of moisture or volatilization of hydrocarbons. After all of the soil required for the in-house analyses was collected, the remaining soil was placed in an I-Chem® bottle and sent to an outside laboratory for nutrient and physical properties analyses.

6.2 Analytical Methods

For the most part, the analytical methods used during this experimental run were the same as those utilized during the second run. Slight modifications were made to the TPH in gas and microbial enumeration analytical methods. The following sections indicate whether an analytical protocol was changed and, if so, descriptions of the modifications are presented.

6.2.1 Oxygen and Carbon Dioxide in Gas Samples

The oxygen and carbon dioxide analytical protocol remained unchanged during this experimental run. Concentrations were measured in gas extracted from each reactor during atmospheric exchanging procedures according to the method described in Section 4.2.1.

6.2.2 Petroleum Hydrocarbons in Gas Samples

Petroleum hydrocarbon concentrations were measured in gas extracted from each reactor during atmospheric exchanging procedures. The GC analytical method described in Section 4.2.2 was used for these analyses; however, the data were collected using both a computer-based data acquisition package (Chrom Perfect version 2.0 for Windows) and an HP 3396 Series II integrator. These acquisition devices were used in place of the HP 3392A integrator used in the two previous experiments to allow for more efficient data storage, retrieval, and analysis.

6.2.3 Petroleum Hydrocarbons in Soil Samples

Petroleum hydrocarbon concentrations in soils from reactors before and after the 30-day incubation period were analyzed using the same GC method used in Experiment #2 as described in Section 4.2.3. The data acquisition method was modified by incorporating the use of the Chrom Perfect® data acquisition program instead of a HP 3396 Series II integrator. This data acquisition program was capable of calculating the concentrations of the 20 compounds listed in the calibration mixture as well as the concentrations of hydrocarbon within boiling point ranges. This allowed for significantly more efficient data analyses.

6.2.4 Soil Moisture

The soil moisture analysis protocol was unchanged from the method used in Experiment #1 as described in Section 4.2.4. There were no modifications to the soil moisture analysis method; however, the dried samples were used in the muffling procedure for organic matter determination as explained in Section 6.2.9.

6.2.5 Soil pH

The sample preparation procedure used in Experiment #2 for measuring soil pH was modified slightly to reduce the potential for water loss during equilibration. The modification included the use of a 20-mL screw-cap scintillation vial in place of the 50-mL glass beaker. In addition to preventing evaporation, the scintillation vial resulted in an increase in the depth of the solution, making the use of the electrode easier than when beakers were used.

6.2.6 Dehydrogenase Activity in Soil Samples

Dehydrogenase activity was measured before inoculation and following the 30-day incubation period. The dehydrogenase activity protocol used during Experiment #2 was modified to increase the incubation period from 48 hours to 5 days. The incubation period was increased to enhance the colorimetric detection of TPF.

6.2.7 Microbial Enumerations in Soil Samples

The microbial enumeration procedure utilized in Experiment #2, as described in Section 5.2.7, was modified to allow for comparisons between the population of JP-4 degraders and the population of total heterotrophic microorganisms. This modification included incorporation of enumeration on total plate count agar into the protocol. In addition, enumerations were conducted at a the midpoint of the experimental run to determine if there were any significant changes in population over the testing period. The following procedures were incorporated into the enumeration protocol.

1. **Preparation of Total Plate Count Agar.** Prepare total plate count agar by adding 23.5 g of Difco's Standard Method agar to 1 L of distilled water. Allow the agar to dissolve by heating and stirring. Dispense 20-mL aliquots of the plate count agar into 30-mL borosilicate glass test tubes. Cap and autoclave to sterilize. Store in a 45°C water bath. Use the same dilutions made for the basal inorganic medium plus JP-4 plates for the total counts plates. Place 1 mL of dilutions into the bottom of a petri dish. Pour one 20-mL test tube of total plate count agar into the petri dish and swirl gently to mix the contents. Allow to solidify. Invert, place in sealed plastic bags, incubate at 25°C in an incubator separate from the JP-4 plates. Incubate for approximately 2 to 4 days. Also plate the amendment inoculum onto the total plate count agar.

2. **Sealing of the Plastic Plate Bags.** Use twist ties instead of tape to seal the plastic bags containing the plates.

3. **Midpoint Bacterial Counts.** Perform microbial enumerations following the above protocol at the midpoint of the experimental period. Take soil samples from the top of the columns by removing the cap and withdrawing an aliquot of soil with a sterilized spatula. Collect the soil samples from the top surface of the soil pile in the biometer flasks by aseptically removing the rubber stopper and withdrawing an aliquot of soil with a sterilized spatula. Place the soil into

test tubes and weigh it; then put it through the serial dilution as described in the enumeration protocol in Section 4.2.7. Reseal the reactors and place them back into the incubator.

6.2.8 Nutrient Concentrations in Soil Samples

Soil samples were sent to A&L Analytical Laboratories, Inc. in Memphis, Tennessee for nutrient analysis as described in the Experimental Design Test Plan (Battelle, 1994). Unlike Experiment #2, the nutrient analysis was necessary as the nutrient addition was increased 100-fold over the first two experiments. The analyses were conducted to measure the increased nutrient concentrations to determine if there was any benefit in biodegradation performance attributed to the increased addition of the nutrients under the sterile amendment condition.

6.2.9 Carbon in Soil Samples

The method used in Experiment #2 for carbon analysis in the initial soil samples for each experimental condition and in soil samples from each reactor following incubation remained unchanged. As with Experiment #2, muffle and gravimetric techniques for organic matter were included in this experiment. This method was included so that the results could be compared with the results from the UIC method. The results from the previous experiment indicated that this method was superior for measuring organic content, and the results from this experiment were to be used to verify this preliminary finding.

6.2.10 Particle Size Distribution

Although the same soil used in the two previous experiments was used for this experiment, soil particle size distribution and textural analyses were conducted during Experiment #3 to verify the assumption that these parameters remained constant during soil storage. The methods used were the same methods described in the Experimental Design Test Plan (Battelle, 1994).

6.2.11 Cation Exchange Capacity

As with the particle size distribution and textural analyses, cation exchange capacity was measured in the soils used in Experiment #3 to verify the assumption that the CEC would not change during soil storage. Samples were sent to A&L Laboratories, Inc. for this analysis using the ammonium saturation method as described in Section 4.2.11.

6.3 Results

The analytical protocols conducted during Experiment #3 as described in Section 6.2 were completed and the data were reduced and evaluated. The results from each protocol are presented in the following sections along with a discussion of the trends between experimental conditions, the added value of the data obtained using the specified protocols, and any method modifications required to enhance the data obtained. The results from the column and biometer flask reactors are presented separately.

6.3.1 Oxygen and Carbon Dioxide in Gas Samples

6.3.1.1 Column Reactors. The results from the Control 2 columns, contaminated soil with sterilized amendment, before oxygen consumption and carbon dioxide production are shown in Figure 36. Control 2 C had the most oxygen consumed and the most carbon dioxide produced among the three columns run under this condition. The total oxygen consumed for Control 2 C was 49.23 mg and the total carbon dioxide produced was 35.97 mg. Control 2 A had the lowest amount of oxygen consumed at a total of 28.90 mg. Control 2 A also had the lowest amount of carbon dioxide produced. After the tenth day, no more carbon dioxide was produced to add to the cumulative production value until sampling on day 24. The cumulative carbon dioxide produced for Control 2 A was 13.83 mg. Control 2 B had a steady increase of oxygen consumed. The cumulative oxygen consumed was 40.10 mg. The carbon dioxide produced in Control 2 B increased slowly throughout the experiment, giving a total of 23.49 mg.

Figure 37 shows the oxygen consumption and carbon dioxide production data from the reactors containing only contaminated soil. Reactor 1 B consumed the most oxygen at a cumulative value of 33.87 mg. The Reactor 1 A and Reactor 1 C data were close throughout the experiment. The Reactor 1 A cumulative oxygen consumed was 22.97 mg, whereas the Reactor 1 C cumulative oxygen consumed was 24.31 mg. Reactor 1 B produced the most carbon dioxide among the three columns with only contaminated soil. The total carbon dioxide produced for Reactor 1 B was 10.16. Both Reactor 1 A and Reactor 1 B had no carbon dioxide produced during the experimental run.

The oxygen and carbon dioxide data from the column reactors containing contaminated soil plus the microbial amendment are shown in Figure 38. Reactor 3 A consumed the most oxygen during the duration of the experiment. The cumulative oxygen consumed in Reactor 3 A was 49.54 mg. Reactor 3 A also produced the most carbon dioxide at 34.06 mg. Reactor 3 C had the second highest amount of oxygen consumed and

carbon dioxide produced, 40.56 mg and 26.09 mg, respectively. Reactor 3 B had the lowest amount of oxygen consumed, 38.70 mg, and the lowest amount of carbon dioxide produced, 21.61 mg.

Figure 39 shows the average cumulative values of oxygen consumed for the three experimental conditions. The Reactor 3 and Control 2 column data were close to each other, showing approximately the same amount of microbial activity occurring. For Reactor 3, contaminated soil with the microbial amendment, the total oxygen consumed was 42.93 mg. For Control 2, contaminated soil, the average cumulative oxygen consumed was 39.41 mg. These values show that addition of the microbial amendment was not beneficial to microbial degradation or microbial activity occurring within the columns. Reactor 1, containing only the contaminated soil, had an average cumulative consumption of oxygen at 27.05 mg.

By comparing the average cumulative oxygen consumption graphs of the biometer flasks (Figure 43) shown in Section 6.3.1.2 with the graphs for the columns (Figure 39), it was determined that the biometer flasks show more microbial activity and are more beneficial to use in subsequent experiments. The average cumulative oxygen consumed for the Control 2 columns was 39.41 mg, but for the Control 2 biometer flasks it was 52.39 mg. The Reactor 1 column had an average cumulative value of 27.05 mg of oxygen consumed, whereas the Reactor 1 biometer flask had 29.10 mg of oxygen consumed. For the Reactor 1 condition, there is not much difference between the columns and the biometer flasks. The Reactor 3 column had an average cumulative oxygen consumption of 42.93 mg and the Reactor 3 biometer flasks had an average total of 67.60 mg.

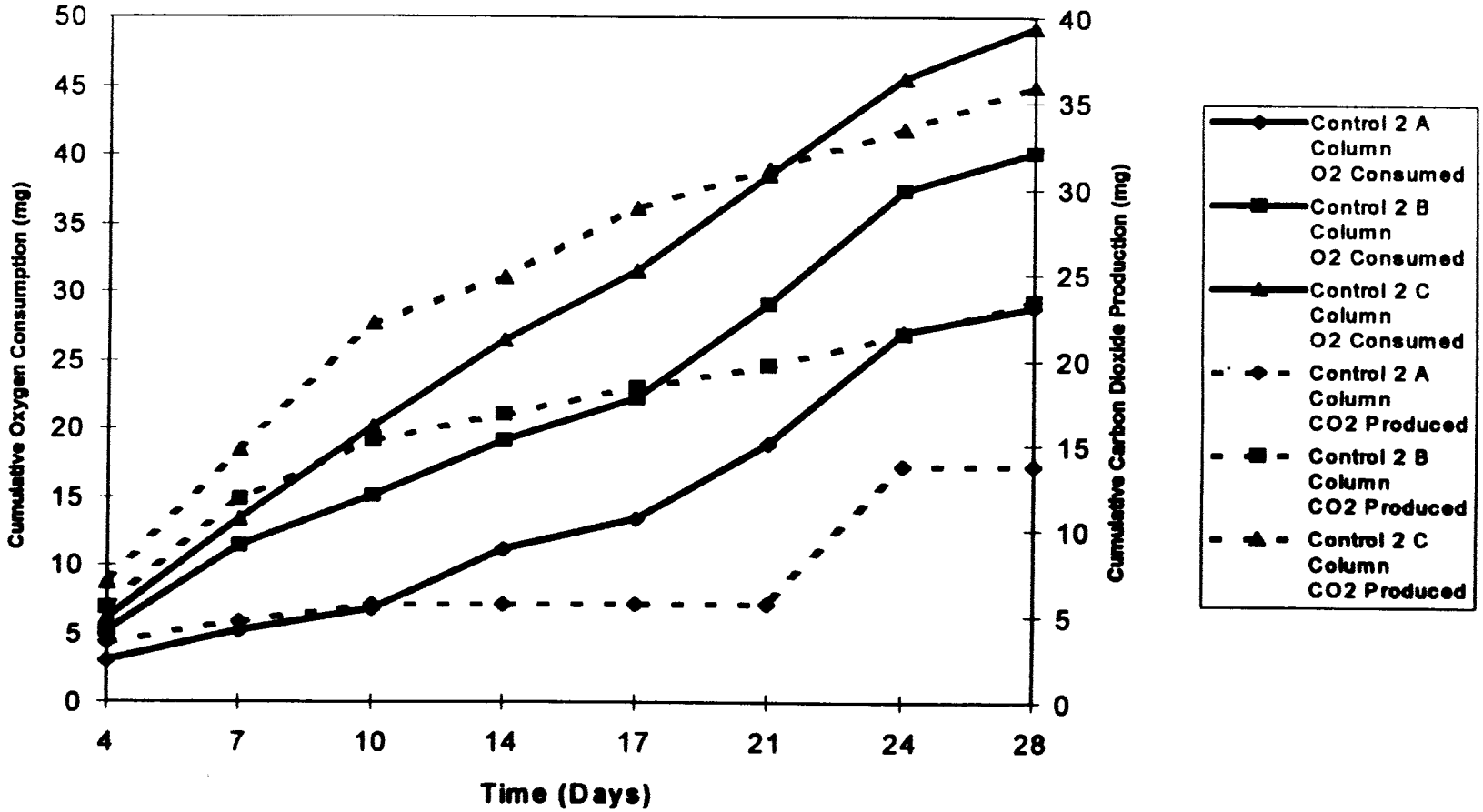

Figure 36. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production (mg) in Columns Reactors Containing Contaminated Soil with Sterilized Amendment During Experiment #3.

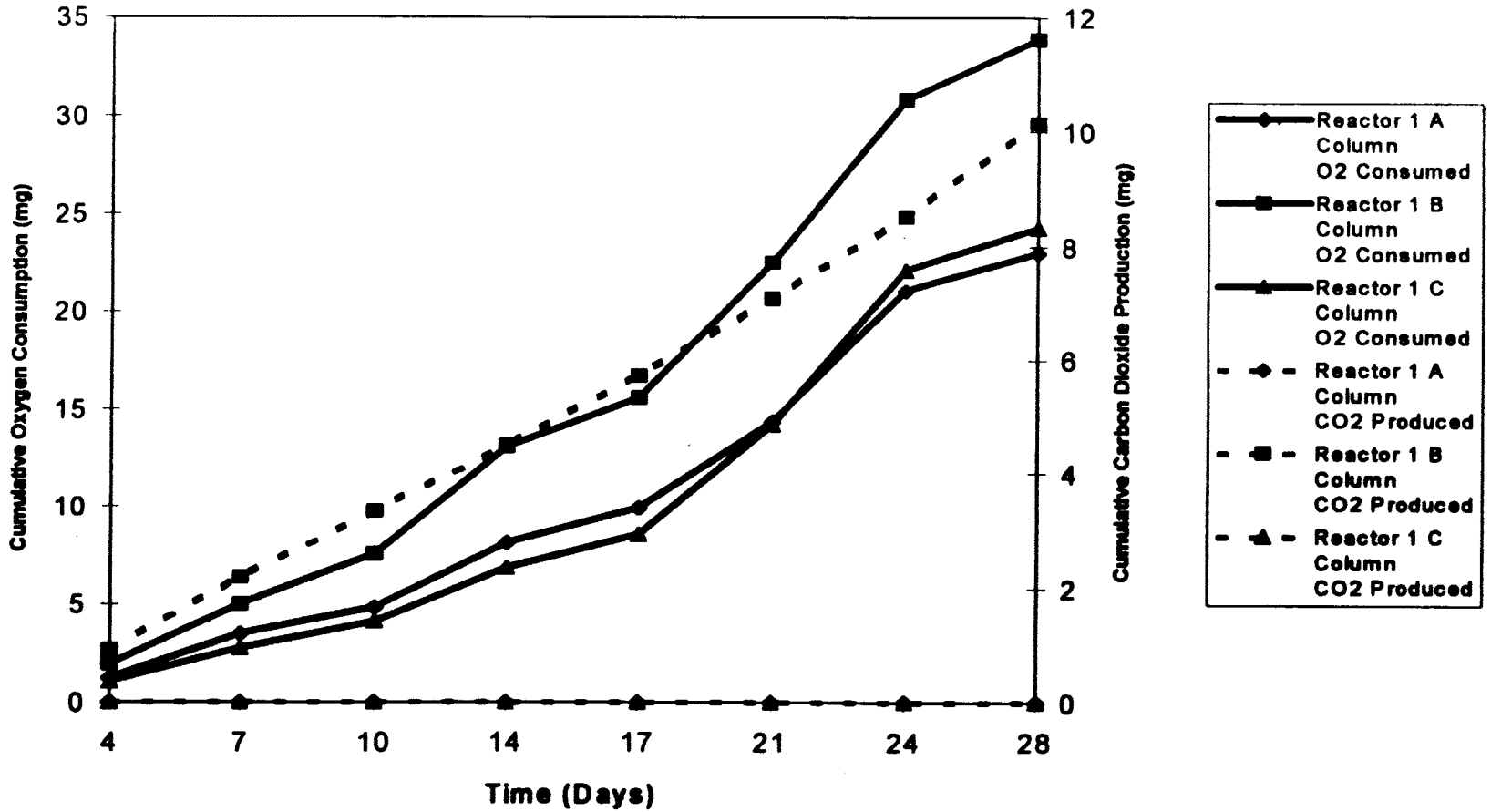

Figure 37. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production (mg) in Columns Reactors Containing Contaminated Soil During Experiment #3.

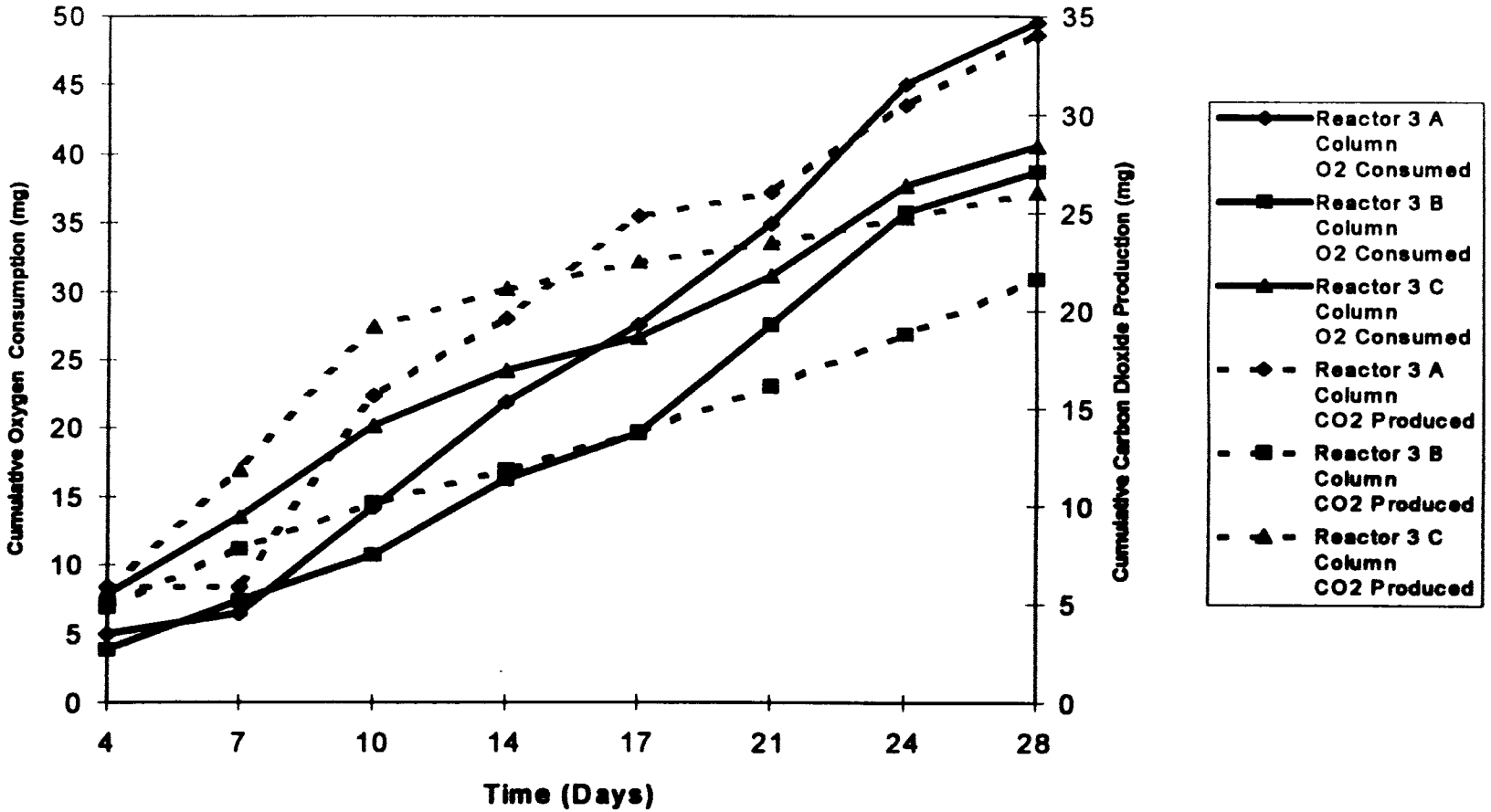

Figure 38. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production (mg) in Columns Reactors Containing Contaminated Soil with Microbial Amendment During Experiment #3.

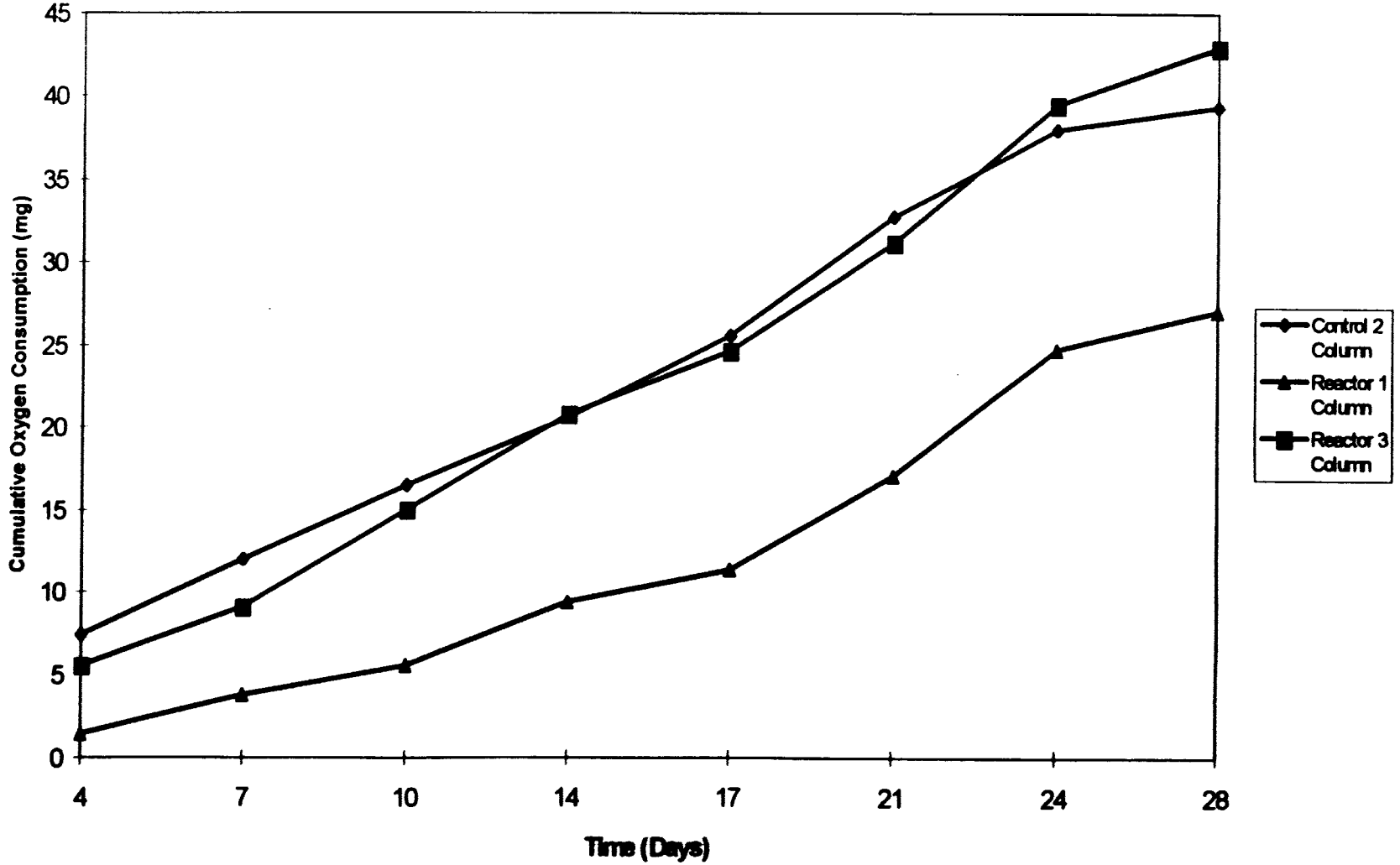

Figure 39. Average Cumulative Oxygen Utilization (mg) in Reactors Containing Soil under all 3 Conditions During Experiment #3

6.3.1.2 Biometer Flask Reactors. Figure 40 shows the oxygen consumed and carbon dioxide produced in gas samples from Control 2 of the biometer flasks, the contaminated soil with the sterilized amendment. The range of oxygen consumed and carbon dioxide produced was the same under this condition during Experiment #3. The Control 2 A flask had the highest amount of oxygen consumed among the three flask reactors with contaminated soil and sterilized amendment. Control 2 B had equal amounts of oxygen consumed as Control 2 A until day 24 when the oxygen consumed in Control 2 B slowed down. The total oxygen consumed for Control 2 A was 59.63 mg, and the total for Control 2 B was 55.59 mg. The carbon dioxide produced in Control 2 A was a few milligrams higher than that in Control 2 B. The total carbon dioxide produced in Control 2 A was 51.92 mg, whereas the cumulative carbon dioxide production for Control 2 B was 49.76 mg. Control 2 C had a much lower cumulation of oxygen consumed and carbon dioxide produced than Control 2 A and Control 2 B. For the first two sampling periods, Control 2 C had no oxygen consumed and no carbon dioxide produced. From day 17 to day 21, the oxygen consumed jumped from 15.93 mg to 26.51 mg, respectively. The total oxygen consumed by the Control 2 C biometer flask was 41.94 mg. The carbon dioxide produced remained low throughout the experiment. The total carbon dioxide produced was 35.97 mg.

The oxygen and carbon dioxide data from Reactor 1 A, B, and C (only contaminated soil) is presented in Figure 41. Reactor 1 B had the most oxygen consumed at a total of 31.37 mg. Reactor 1 A and Reactor 1 C had almost equal amounts of oxygen consumed, 28.73 mg and 27.19 mg , respectively. The carbon dioxide production was approximately five times lower than that of the contaminated soil with sterilized amendment (Control 2). Reactor 1 B had the highest production of carbon dioxide at 11.26 mg. Reactor 1 A had 8.62 mg of carbon dioxide produced, whereas Reactor 1 C had the lowest amount of carbon dioxide produced at 4.13 mg. Reactor 1 C had no production of carbon dioxide until sampling on day 17 of the experimental period.

Figure 42 shows the oxygen consumption and carbon dioxide production for the contaminated soil with the microbial amendment. The data are very close for the three biometer flask reactors with this experimental condition. Reactor 3 A, B, and C also had higher rates of oxygen consumption and carbon dioxide production than the other two experimental conditions. This phenomenon is what we were looking for with the microbial amendment addition. The total oxygen consumption for Reactor 3 A was 64.54 mg, Reactor 3 B was 71.99 mg, and Reactor 3 C was 66.26 mg. Reactor 3 A had a cumulative carbon dioxide production of 54.98 mg, Reactor 3 B had 58.22 mg, and Reactor 3 C had 60.62 mg.

Figure 43 compares the average oxygen consumption of Control 2, Reactor 1, and Reactor 3. Reactor 3 had the average highest amount of oxygen consumed at 67.60 mg. Reactor 3 was the contaminated soil with the microbial amendment addition. More microbial activity was occurring in these biometer flasks than the other two conditions biometer flasks. Control 2, contaminated soil with sterilized amendment addition, had the second highest amount of oxygen consumed, with a averaged total value of 52.39 mg. Reactor 1 had an average total of 29.10 mg of oxygen consumed, the lowest among the three experimental conditions.

6.3.2 Petroleum Hydrocarbons in Gas Samples

6.3.2.1 Column Reactors. The data for the cumulative TPH in gas samples from contaminated soil with sterile amendment are graphed in Figure 44. The three columns with the contaminated soil and sterilized microbial amendment had the same cumulative TPH until sampling on day 17. On day 17, Control 2 A added only 0.14 µg to the cumulative TPH, whereas Control 2 B added 4.64 µg and Control 2 C added 2.74 µg. The following 11 days of the experiment showed a steady increase of the cumulative TPH for all of the columns. The cumulative TPH for Control 2 A was 24.87 µg, for Control 2 B was 33.15 µg, and for Control 2 C was 33.68 µg.

Figure 45 shows the cumulative TPH for Reactor 1 A, Reactor 1 B, and Reactor 1 C. Reactor 1 A had the highest cumulative TPH among the three columns with contaminated soil. The cumulative TPH for Reactor 1 A was 38.60 µg. Reactor 1 B and Reactor 1 C had similar cumulative TPH values, 27.38 µg and 27.68 µg, respectively. The cumulative TPH values for the Reactor 1 columns are in the same range as the cumulative TPH values for the Control 2 columns.

The cumulative TPH data for the contaminated soil with the microbial amendment are shown in Figure 46. Reactor 3 B had twice as much cumulative TPH as Reactor 3 A and Reactor 3 C. Reactor 3 A and Reactor 3 B began with the same amount of TPH, yet while the TPH was being quickly depleted in Reactor 3 A, the TPH was being depleted slightly in Reactor 3 B. Reactor 3 A had a cumulative TPH of 21.99 µg and Reactor 3 B had 41.14 µg of cumulative TPH. Reactor 3 C began with half as much TPH as Reactor 3 A and Reactor 3 B. However, this TPH was not depleted as much as the TPH in Reactor 3 A. The cumulative TPH of Reactor 3 C was 19.79 µg.

6.3.2.2 Biometer Flask Reactors. The cumulative TPH values in gas sample data for the contaminated soil with sterile amendment in biometer flasks are graphed in Figure 47. The Control 2 B flask contained the highest cumulative TPH among the three flasks with the contaminated soil, with cumulative

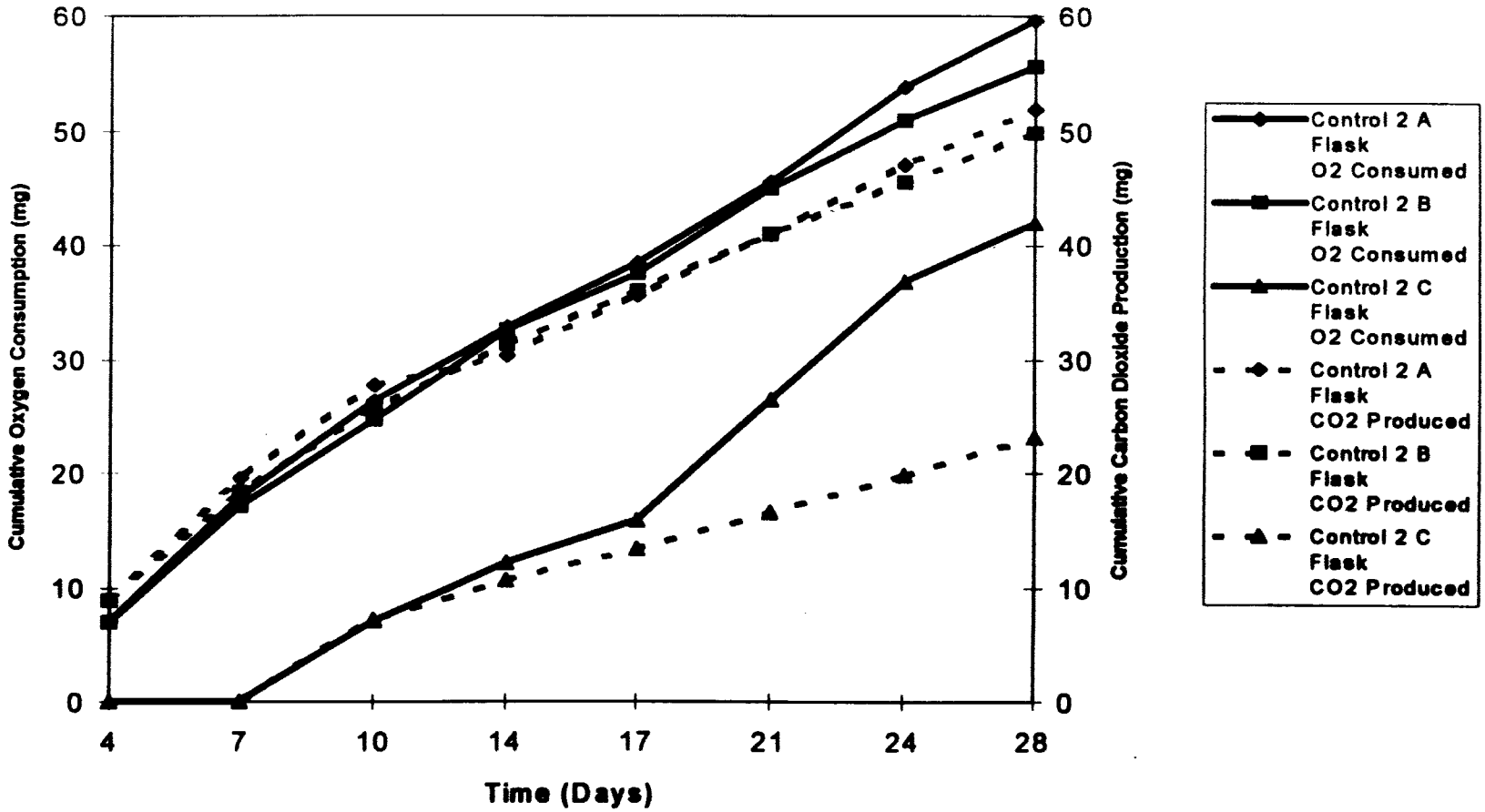

Figure 40. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production (mg) in Biometer Flask Reactors Containing Contaminated Soil with Sterilized Amendment During Experiment #3.

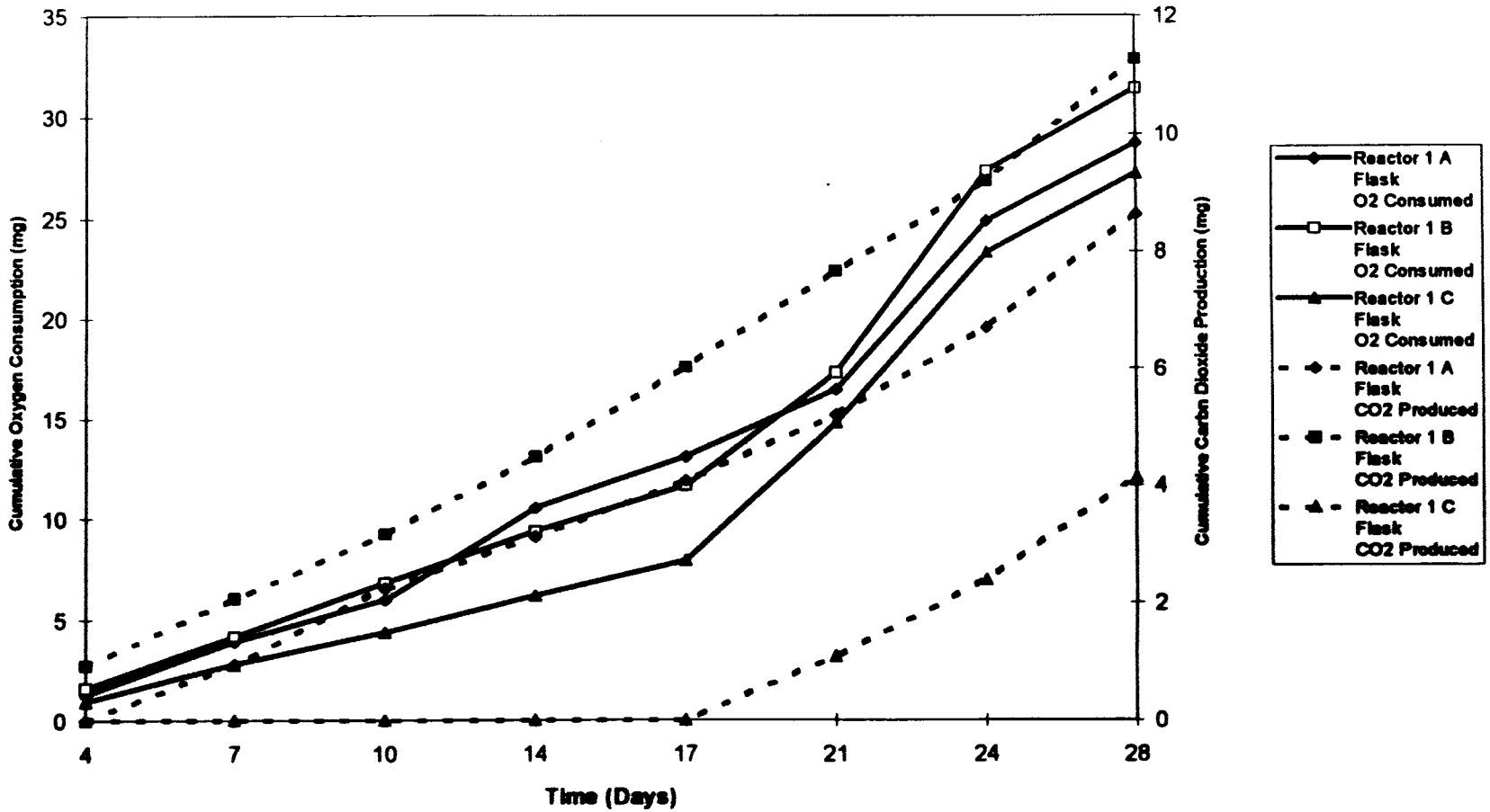

Figure 41. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production (mg) in Biometer Flask Reactors Containing Contaminated Soil During Experiment #3.

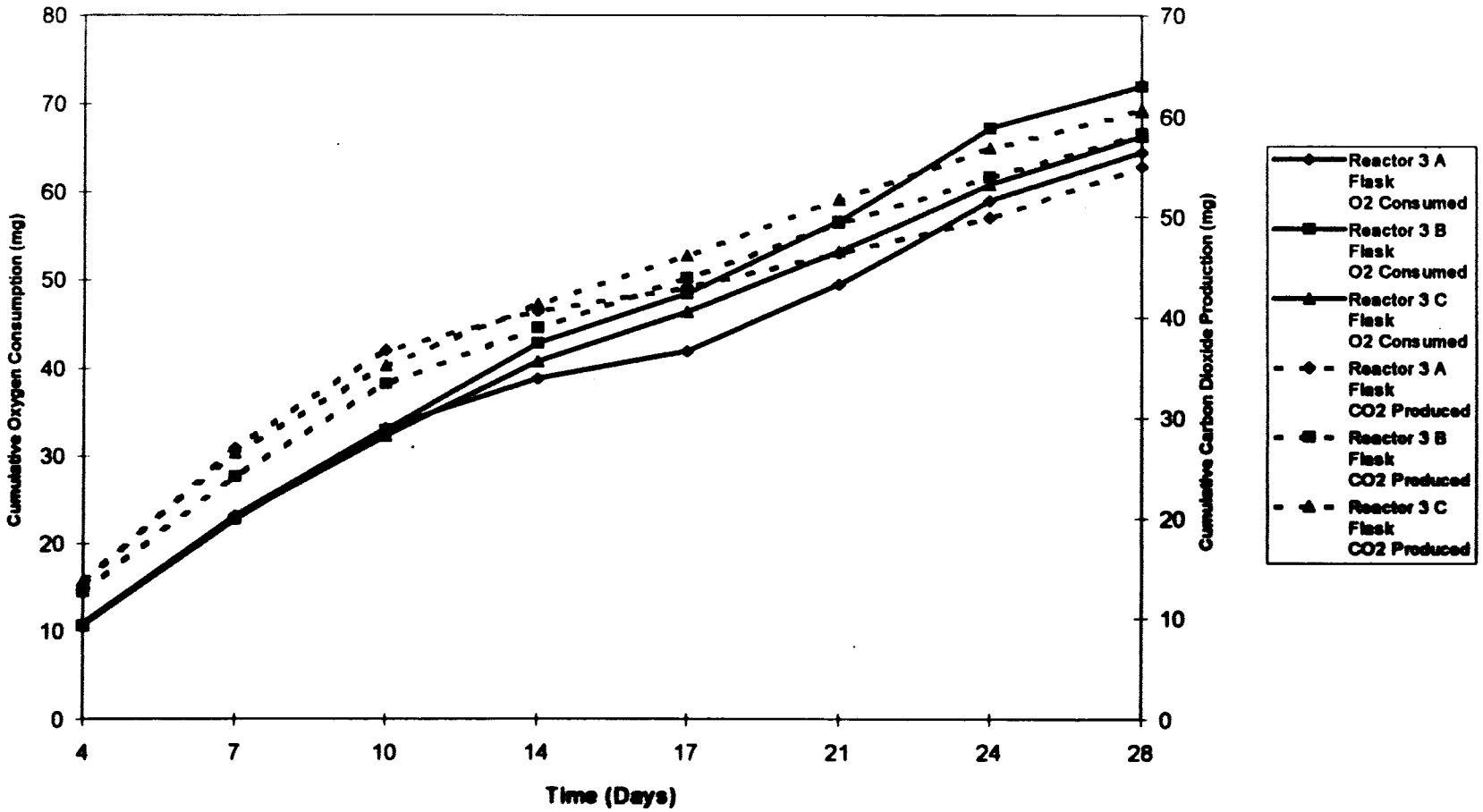

Figure 42. Cumulative Oxygen Utilization and Cumulative Carbon Dioxide Production (mg) in Biometer Flask Reactors Containing Contaminated Soil with Microbial Amendment During Experiment #3.

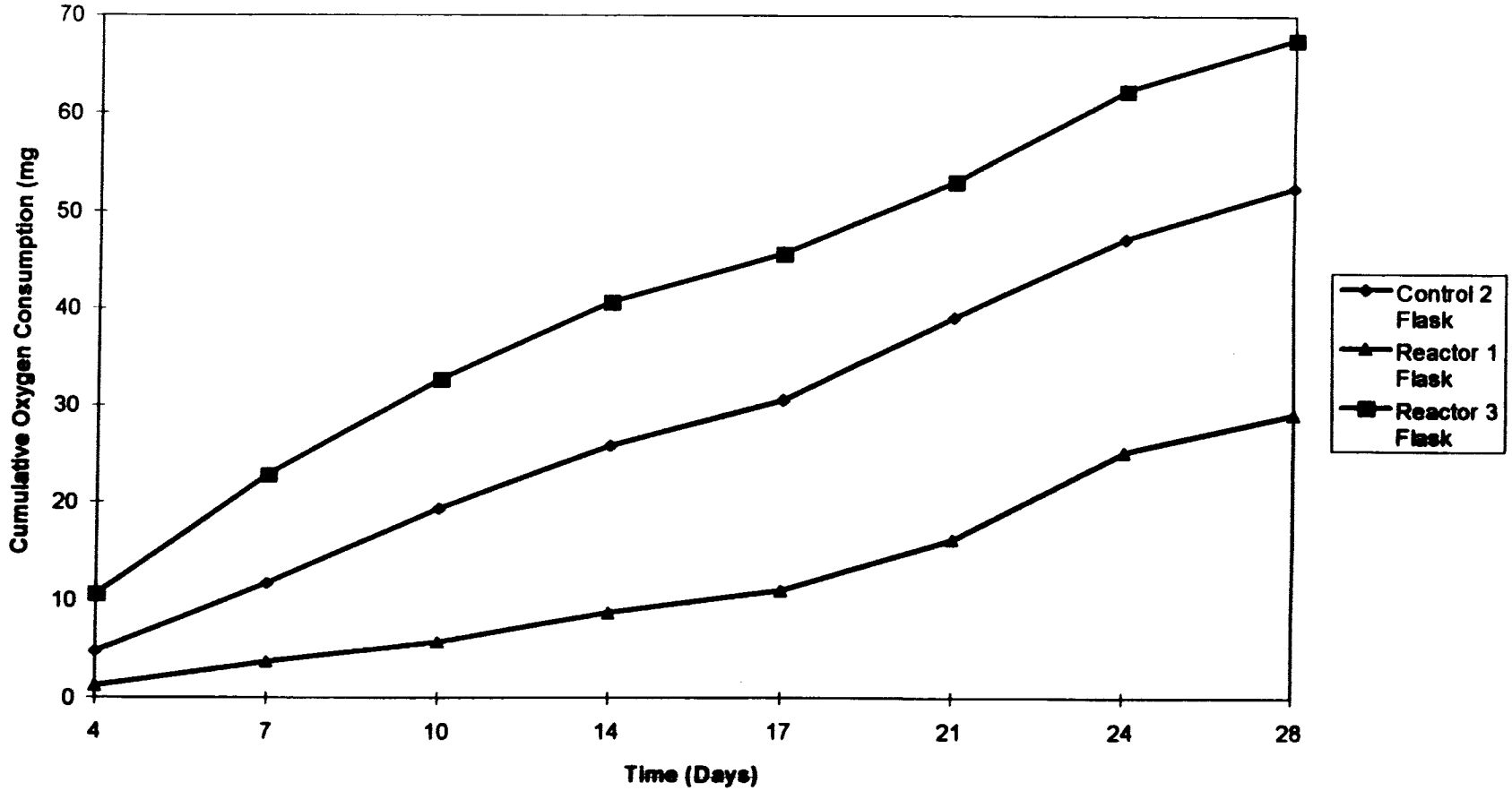

Figure 43. Average Cumulative Oxygen Utilization in Biometer Flask Reactors Containing Soils Under the 3 Conditions During Experiment #3.

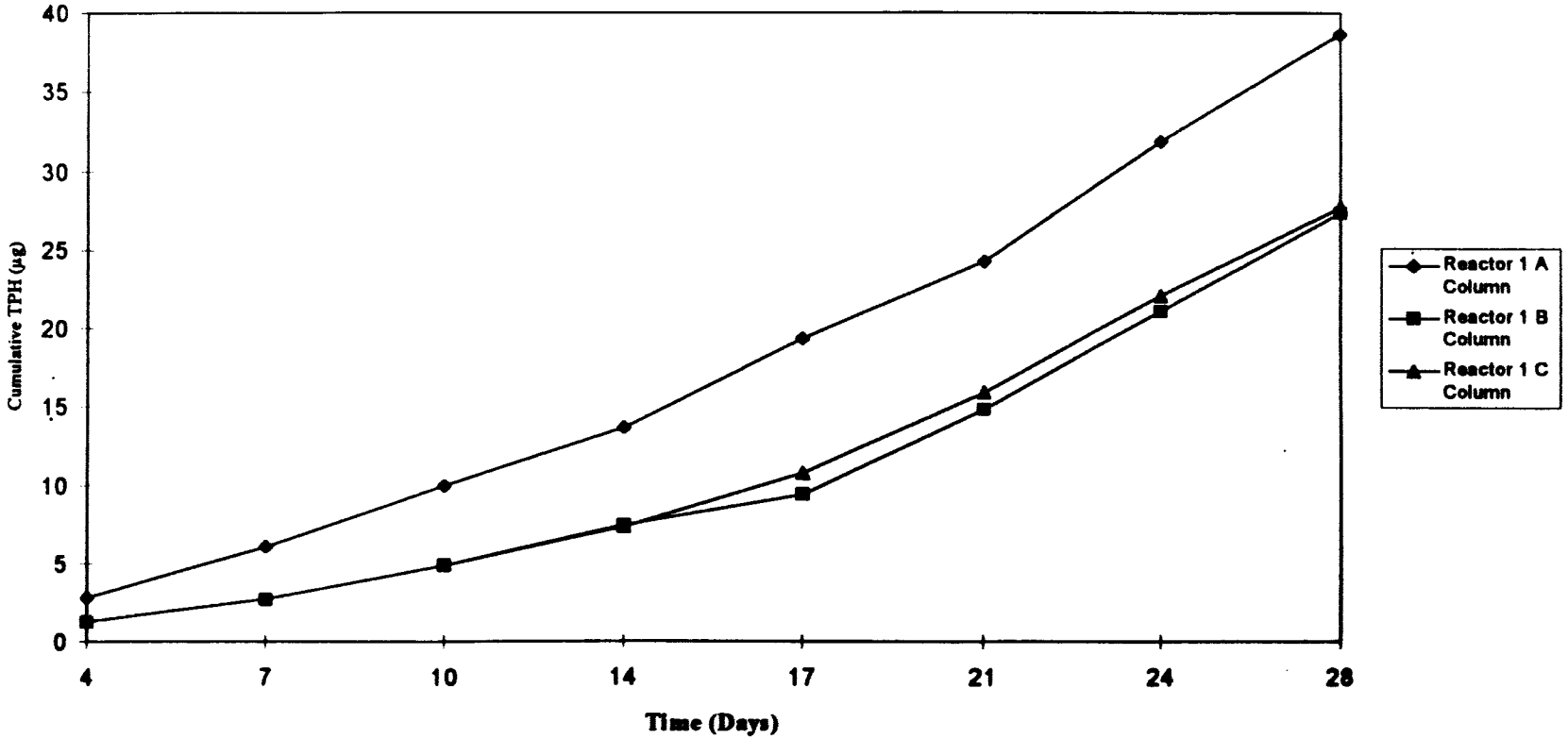

Figure 45. Cumulative TPH (µg) Removed from Column Reactors Containing Contaminated Soil During Atmosphere Exchanging in Experiment #3.

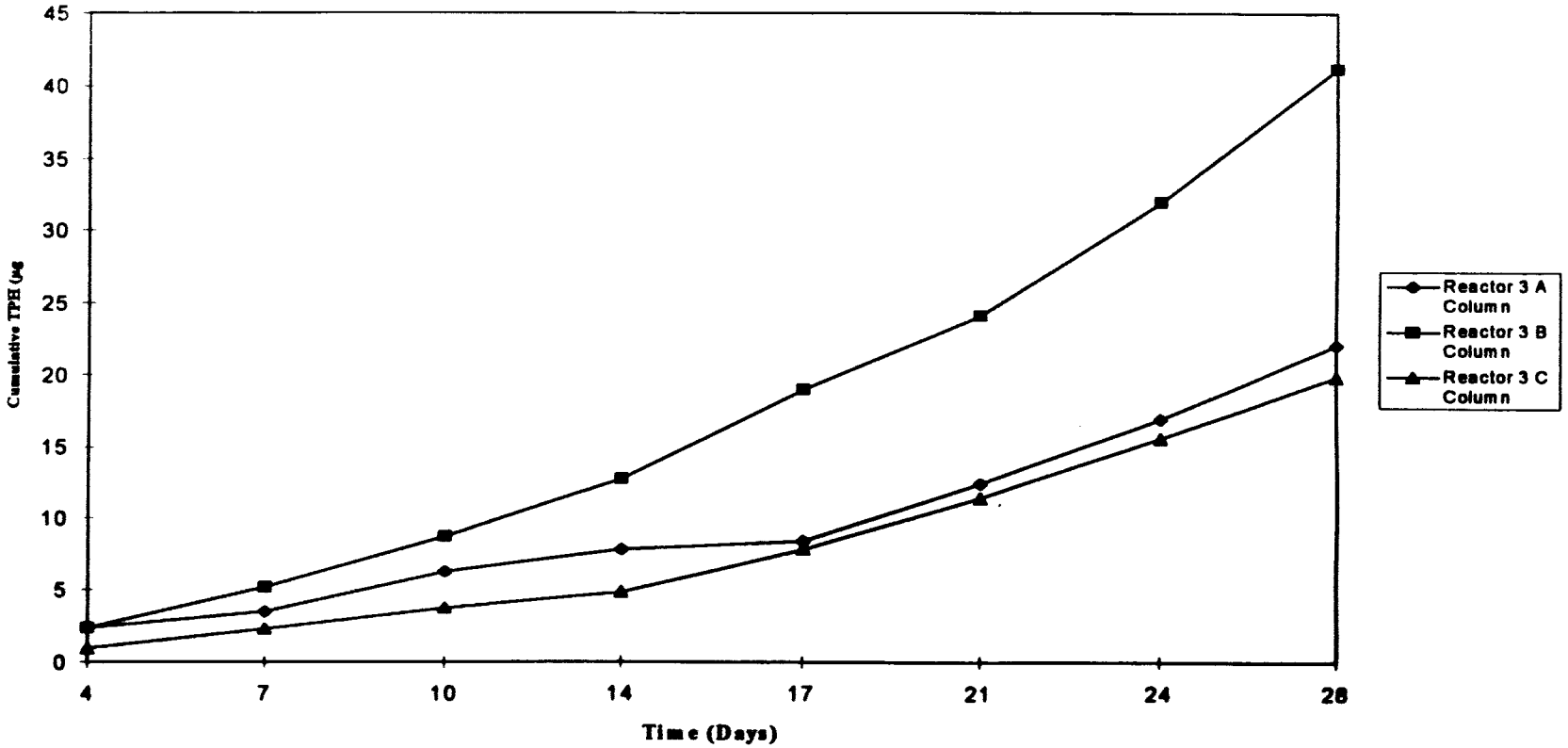

Figure 46. Cumulative TPH (µg) Removed from Column Reactors Containing Contaminated Soil with Microbial Amendment During Atmosphere Exchanging in Experiment #3.

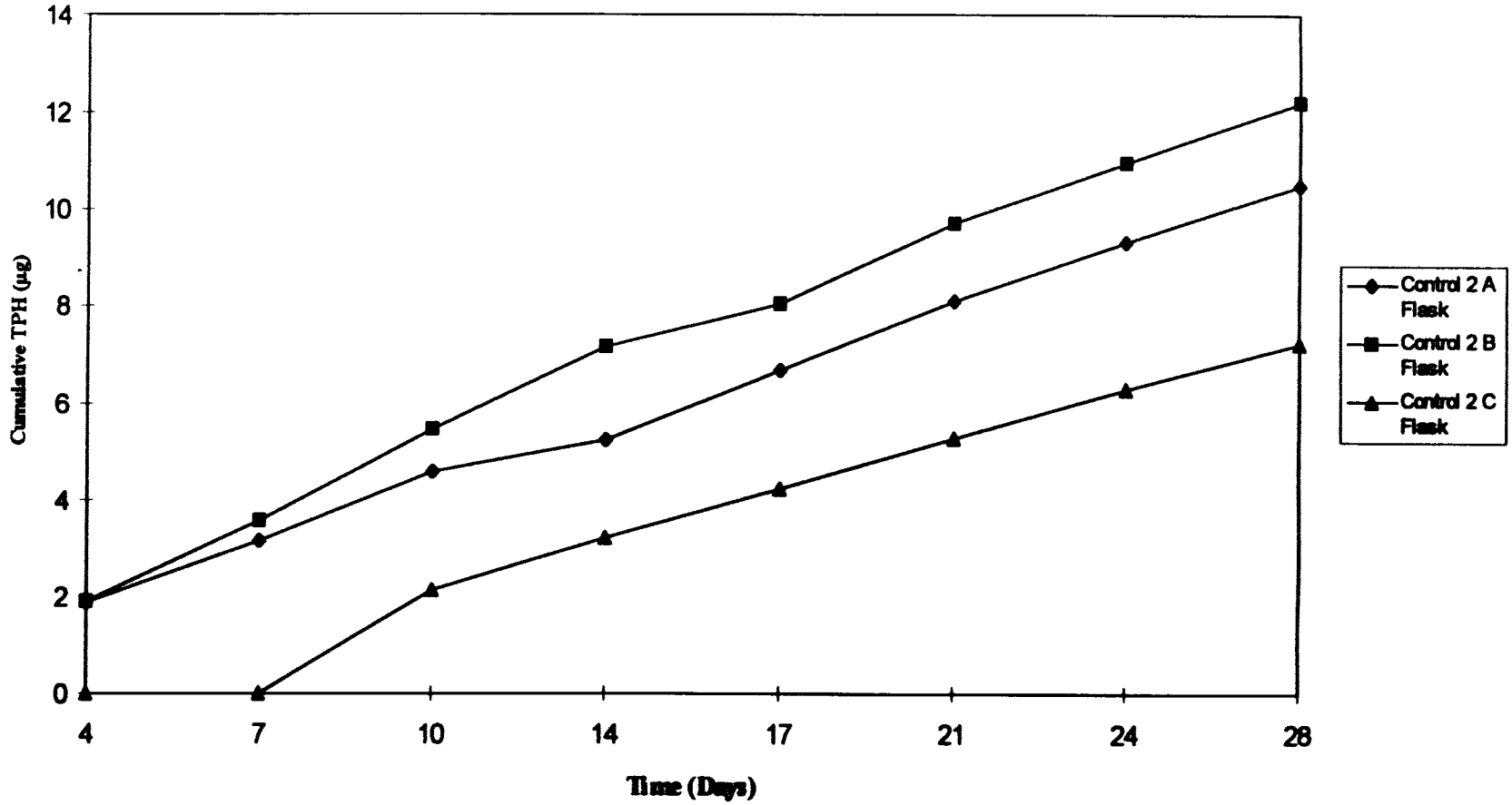

Figure 47. Cumulative TPH (µg) Removed from Biometer Flask Reactors Containing Contaminated Soil with Sterile Amendment During Atmosphere Exchanging in Experiment #3.

TPH of 12.20 μg. Control 2 A had a cumulative TPH of 10.50 μg and Control 2 C had 7.24 μg of cumulative TPH. The Control 2 flasks had an average of 9.98 μg of cumulative TPH. The Control 2 columns had an average of 30.57 μg of cumulative TPH. The columns began with approximately twice as much TPH as the biometer flasks.

Figure 48 shows the cumulative TPH in gas samples for the contaminated soil. Reactor 1 A had a cumulative TPH of 13.88 μg, Reactor 1 B had 16.85 μg, and Reactor 1 C had 11.91 μg of cumulative TPH. The Reactor 1 biometer flasks had an average of 14.21 μg cumulative TPH, whereas the Reactor 1 columns had an average of 31.22 μg cumulative TPH.

The cumulative TPH values for the contaminated soil with the microbial amendment addition are shown in Figure 49. The three flasks had equal amounts of cumulative TPH until day 14. When Reactor 3 B added only 0.05 μg of TPH to its total. Reactor 3 B had the lowest cumulative TPH at 7.47 μg. The cumulative TPH for Reactor 3 A was 10.86 μg and for the Reactor 3 B biometer flask the cumulative TPH was 11.05 μg. The average cumulative TPH for the Reactor 3 columns was 27.64 μg, whereas for the Reactor 3 biometer flasks it was 9.79 μg.

6.3.3 Petroleum Hydrocarbons in Soil Samples

6.3.3.1 Biometer Flask Reactors. Table 20 shows the TPH mass for the biometer flasks in Experiment #3. A negative average change indicates a final mass of petroleum hydrocarbons that is less than the original mass values. The control, contaminated soil with sterilized amendment addition, decreased its hydrocarbon mass by 18.93%. The contaminated soil showed only a positive average change, indicating more mass of hydrocarbons at the final analysis than at the beginning. Reactor 3 C was the outlier, with a mass reading of 3.44 mg. The TPH mass for Reactor 3 A and for Reactor 3 B were essentially equal to the initial value of 2.21 mg for the Reactor 3 condition. The contaminated soil with the microbial amendment addition had the greatest change, reducing the TPH mass by 26.16%.

The bar graphs in Figures 50, 51, and 52 show the 20 petroleum hydrocarbon compound masses for the conditions carried out in Experiment #3. Control 2, contaminated soil with sterile amendment, initially contained 10 petroleum hydrocarbon compounds. Three of these compounds were depleted by the end of the experiment. The *n*-dodecane and *n*-pentadecane appeared in the final analyses, but not in the initial analysis. The mass of the seven remaining compounds initially present decreased. Figure 50 depicts the masses of petroleum hydrocarbon compounds in Control 2.

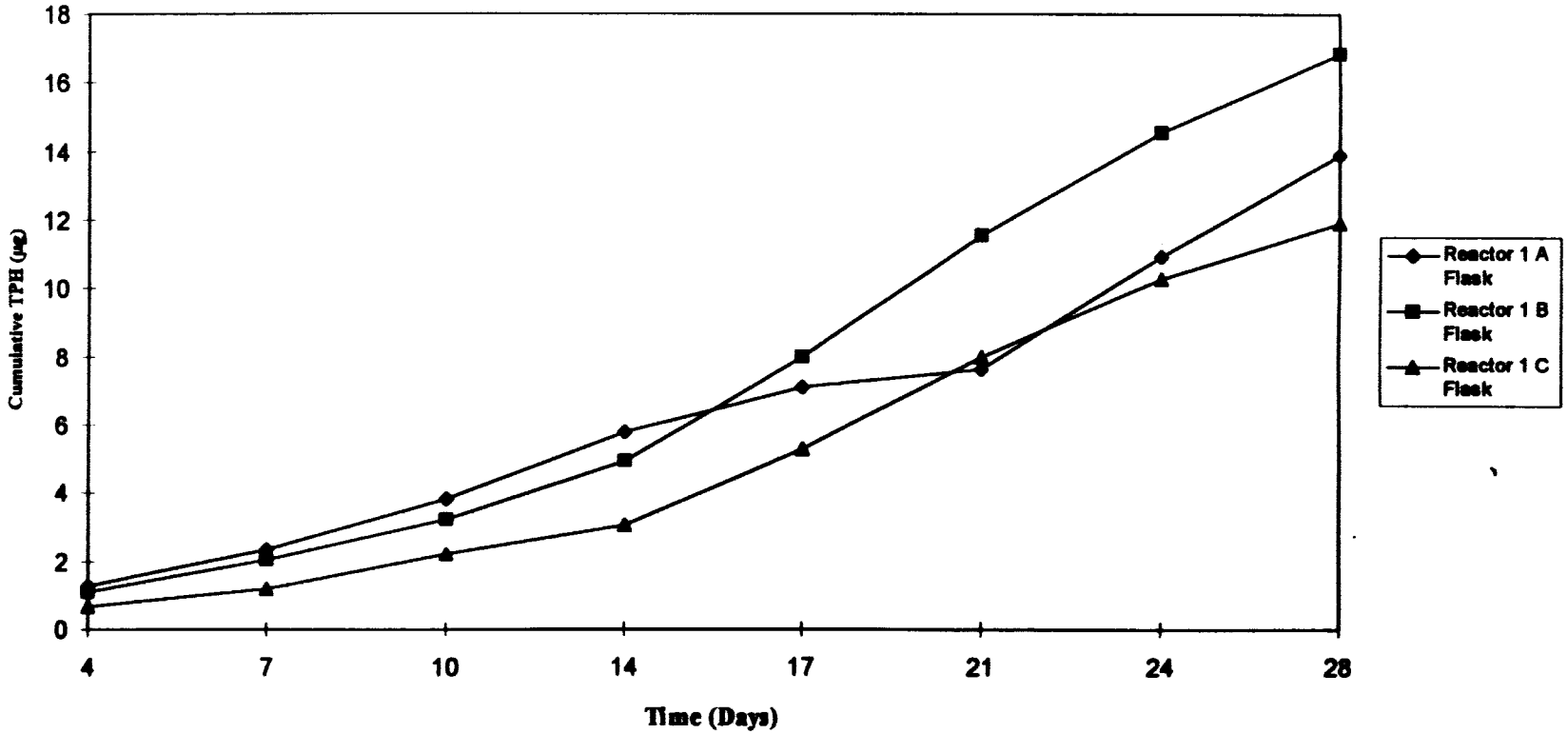

Figure 48. Cumulative TPH (µg) Removed from Biometer Flask reactors Containing Contaminated Soil During Atmosphere Exchanging in Experiment #3.

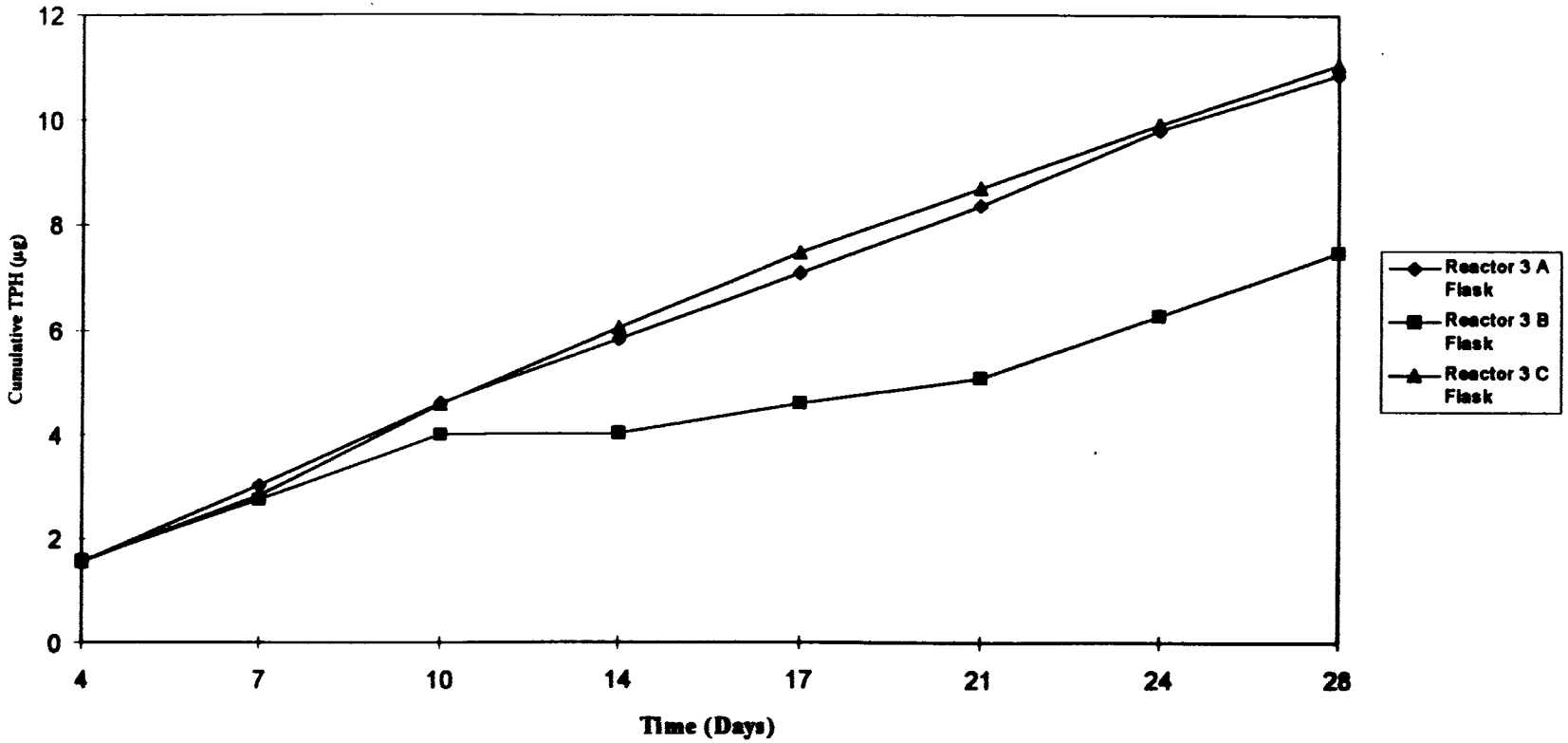

Figure 49. Cumulative TPH (µg) Removed from Biometer Flask Reactors Containing Contaminated Soil with Microbial Amendment During Atmosphere Exchanging in Experiment #3.

Table 20. Mass of Total Petroleum Hydrocarbons (mg) in Soil Samples from Biometer Flasks.

Contaminated Soil with No Amendment				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
2.80	2.17	1.97	2.66	-18.93
Contaminated Soil with Sterilized Amendment Addition				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
2.21	2.27	2.24	3.44	19.91
Contaminated Soil with Amendment Addition				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
2.37	1.75	1.87	1.63	-26.16

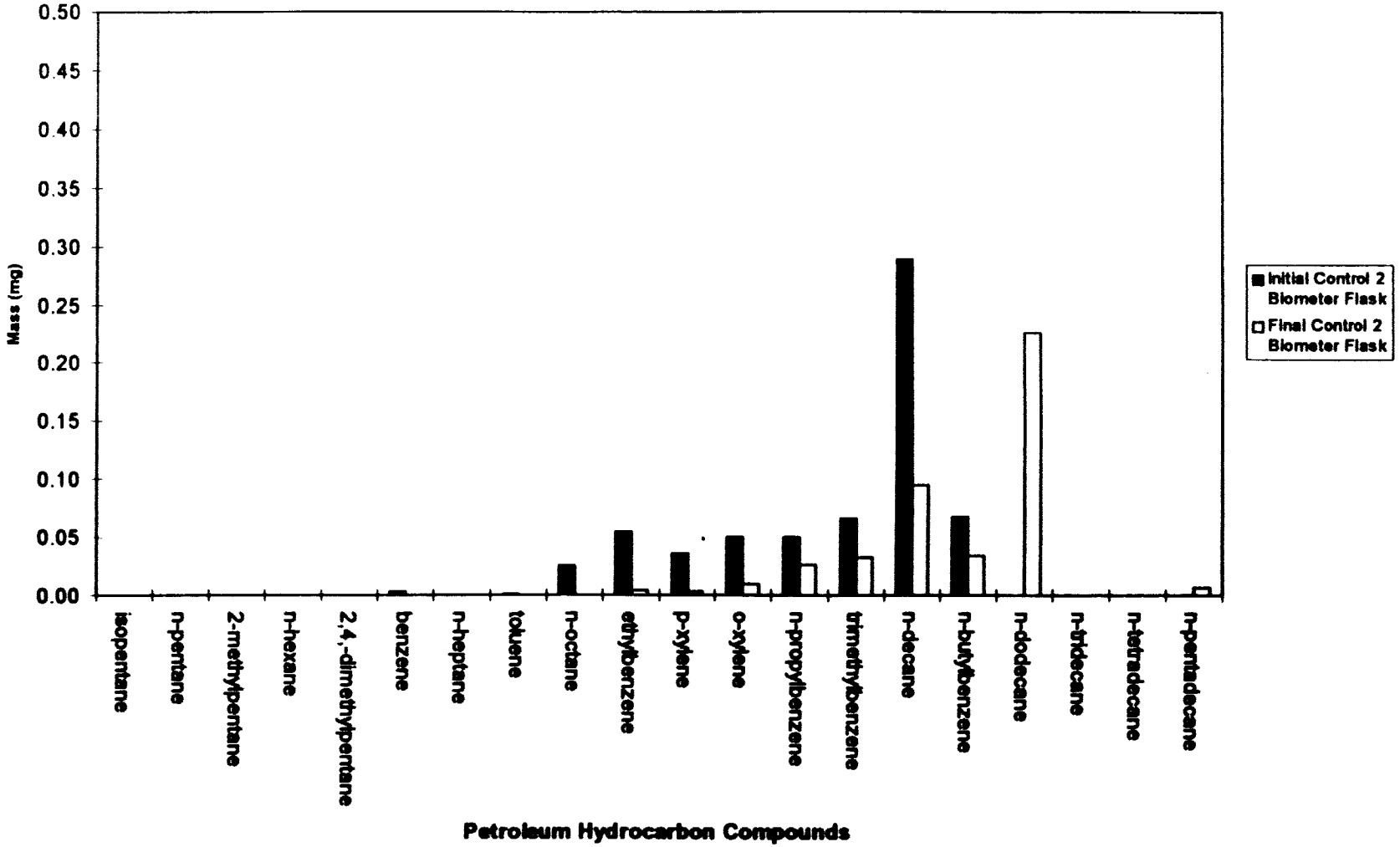

Figure 50. Initial and Average Final Mass of Petroleum Hydrocarbon Compounds (mg) in Biometer Flask Reactor Containing Contaminated Soil with Sterilized Amendment During Experiment #3.

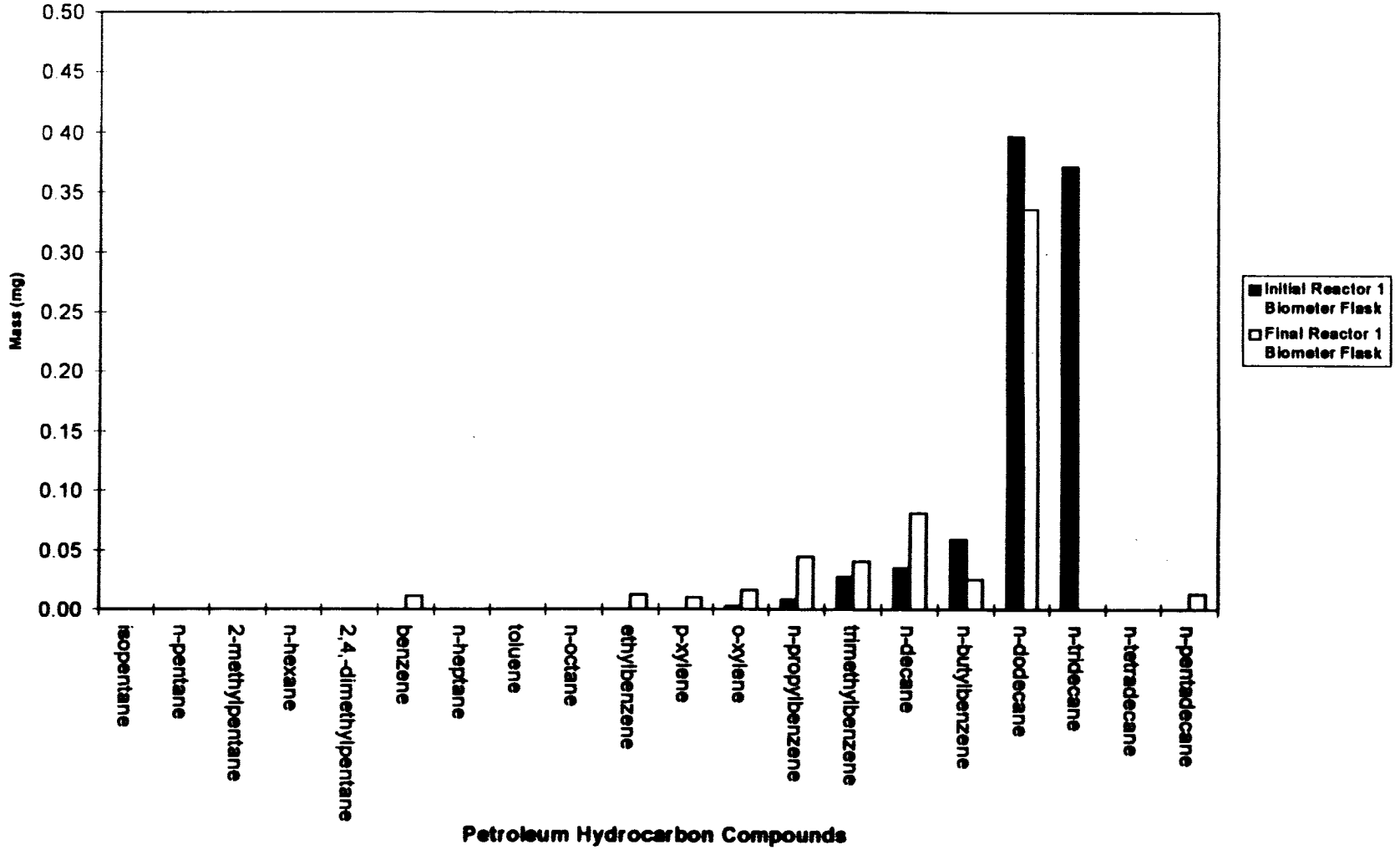

Figure 51. Initial and Average Final Mass of Petroleum Hydrocarbon Compounds (mg) in Biometer Flask Reactors Containing Contaminated Soil During Experiment #3.

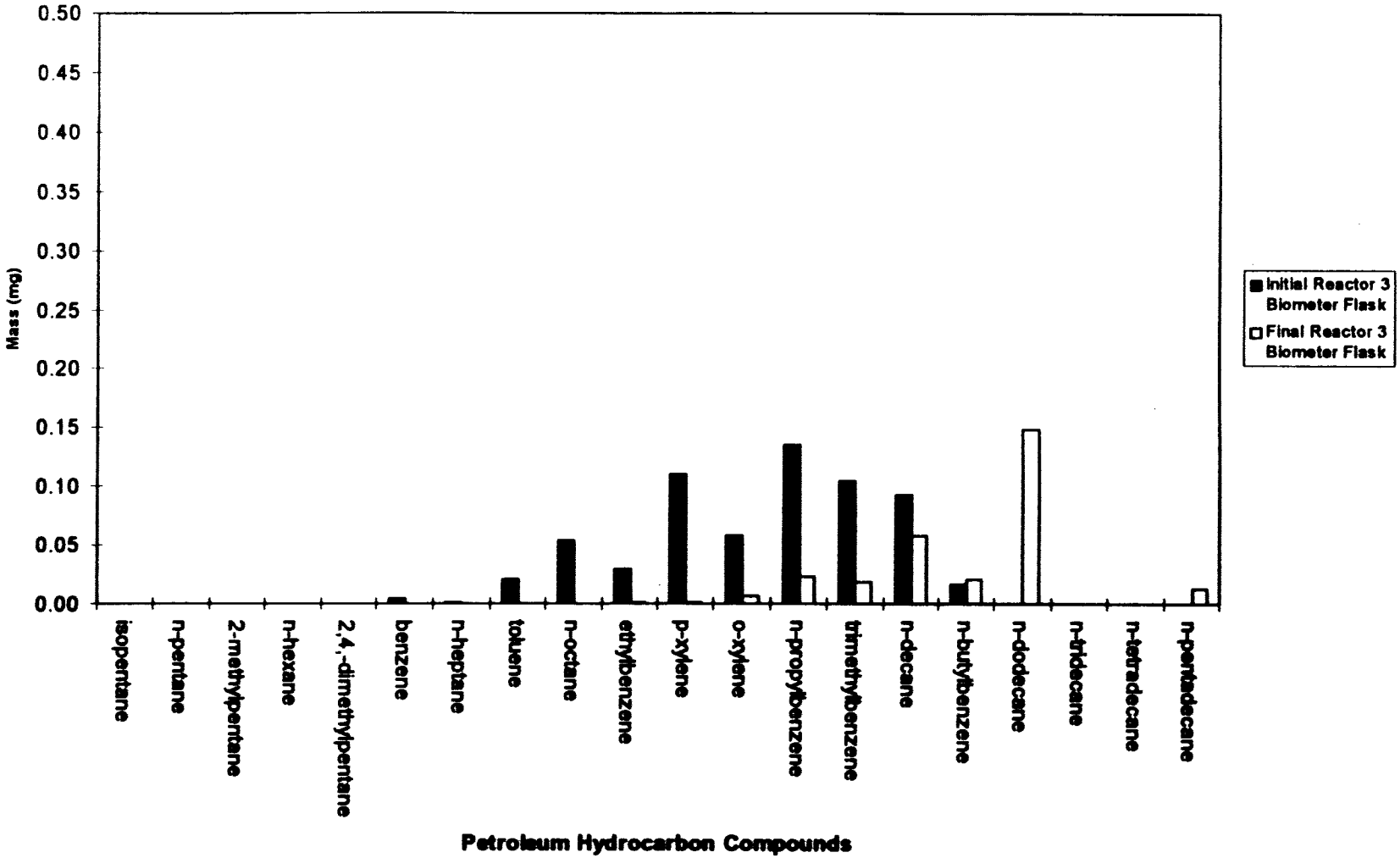

Figure 52. Initial and Average Final Mass of Petroleum Hydrocarbon Compounds (mg) in Soil Samples from Biometer Flask Reactors Containing Contaminated Soil with Microbial Amendment During Experiment #3.

6.3.3.2 Column Reactors. Table 21 exhibits the TPH mass for soil samples from columns in Experiment #3. All conditions show a positive average change, which means the final mass of petroleum hydrocarbons is greater than the original values. For the contaminated soil with sterilized amendment, Control 2, the Reactor B mass decreased, where as the Reactor A and Reactor B mass increased. The average change was 1.43%.

The TPH mass in the reactors with only the contaminated soil increased by 15.99%. The soil samples from Reactor 1 C contained approximately the same mass of hydrocarbons as the initial samples (increase of 2%). The mass of Reactor 1 A increased by 6%. Reactor 1 B increased its mass by 40%, going from 2.21 mg to 3.08 mg. The columns with contaminated soil with microbial amendment addition changed the most, just as the biometer flask changed the most. However, the columns had a positive average change, interpreted as more hydrocarbon mass at the conclusion of the experiment than at the beginning of the experiment.

The bar charts shown in Figures 53, 54, and 55 display the masses of the nineteen petroleum hydrocarbon compounds that are present in the soil samples during the third experiment. The soil from the columns containing the sterilized amendment (Figure 53) had the same initial trends as the contaminated soil from the biometer flasks (Figure 50) for this condition because the soils in the reactors came from the same homogeneous batch. At the termination of the experiment, the benzene, toluene, and *n*-octane previously present in the column samples were depleted. The compound *n*-dodecane was detected at the end of the experiment along with 0.002 mg of *n*-pentadecane, although they were not present initially in the soil samples.

The soil samples from the columns containing only contaminated soil (Figure 54) contained the same petroleum hydrocarbon compounds as the soil in the biometer flasks of this condition (Figure 51) because the soils came from both reactor types came from the same soil stock. The *n*-dodecane was reduced more in the columns than in the biometer flasks. The *n*-decane for the columns had three times the mass in the final analysis as in the initial analysis.

The initial soil samples from the column and biometer flask reactors containing contaminated soil plus microbial amendment were identical because they came from the same original sampling bag. Overall, the mass of petroleum hydrocarbons in the columns decreased (Figure 55), however, the reduction in the biometer flasks (Figure 52) was greater. The major reason the final values are so high for the columns is the 0.342 mg of *n*-dodecane and the 0.276 mg of *n*-decane.

Table 21. Mass of Total Petroleum Hydrocarbon (mg) in Soil Samples from Columns.

Contaminated Soil with No Amendment				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
2.80	3.21	2.17	3.14	1.43
Contaminated Soil with Sterilized Amendment Addition				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
2.21	2.35	3.08	2.26	15.99
Contaminated Soil with Amendment Addition				
Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
2.37	2.91	4.03	2.35	30.80

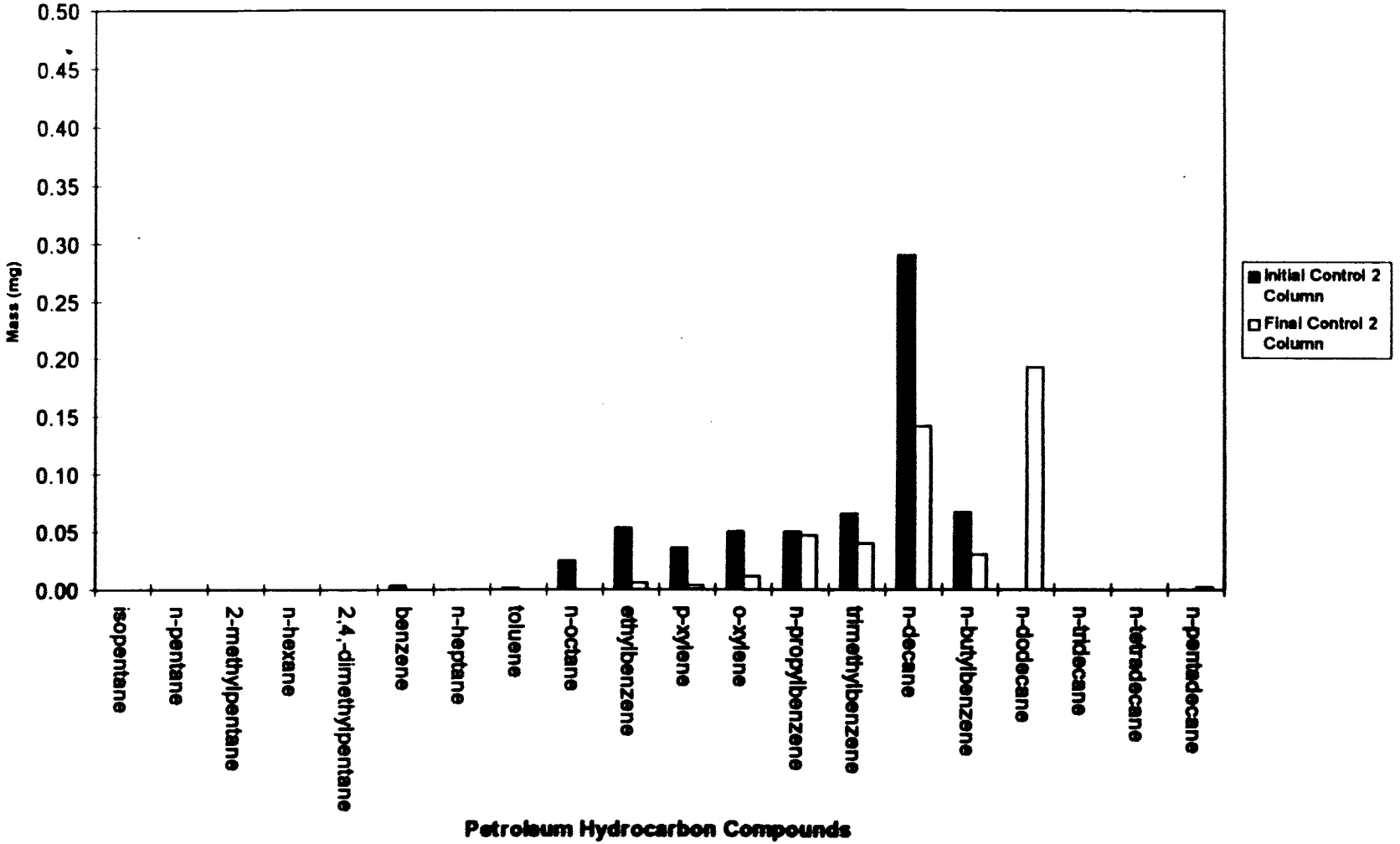

Figure 53. Initial and Average Final Mass of Petroleum Hydrocarbon Compounds (mg) in Column Reactors Containing Contaminated Soil with Sterilized Amendment During Experiment #3.

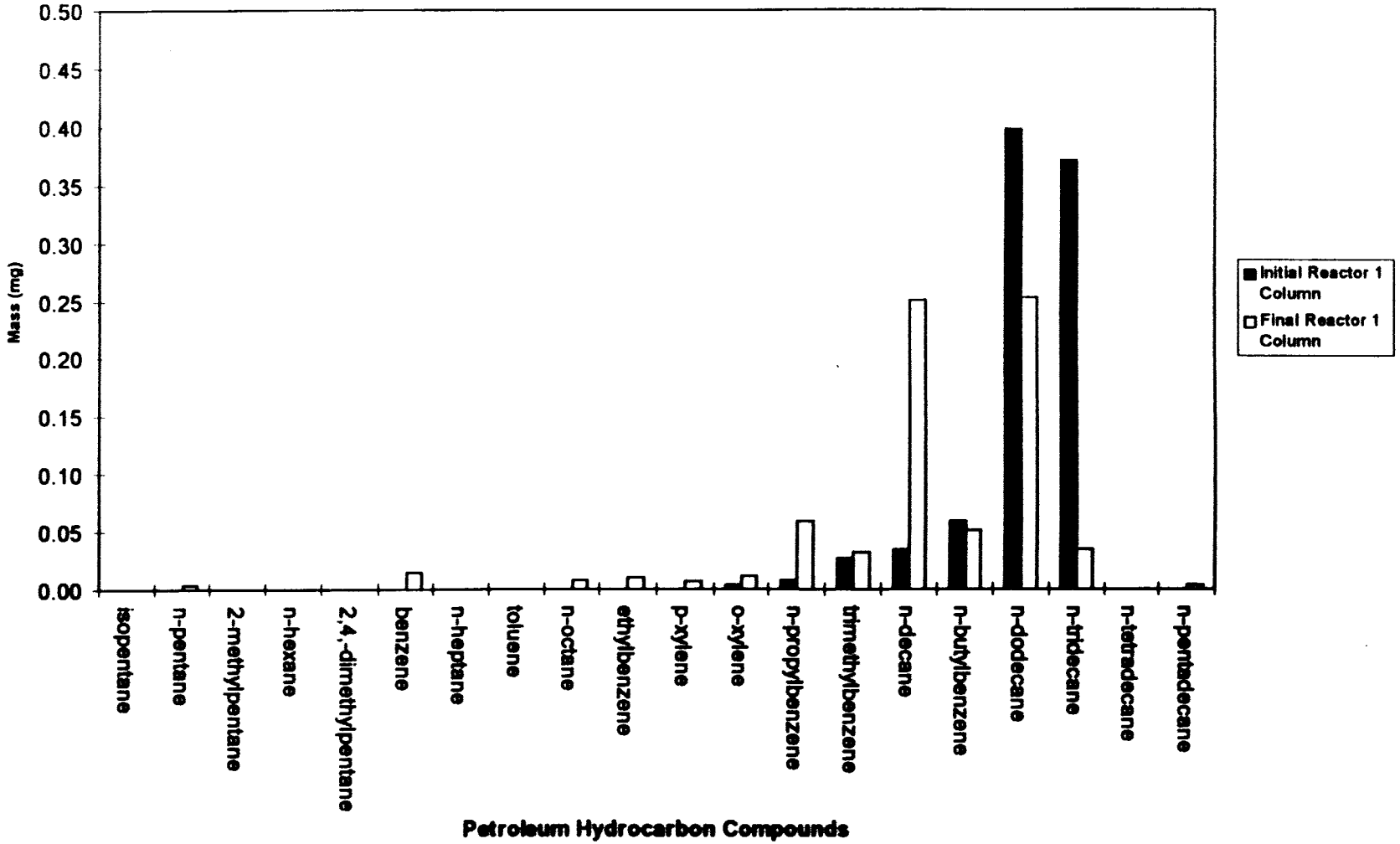

Figure 54. Initial and Average Final Mass of Petroleum Hydrocarbon Compounds (mg) in Column Reactors Containing Contaminated Soil During Experiment #3.

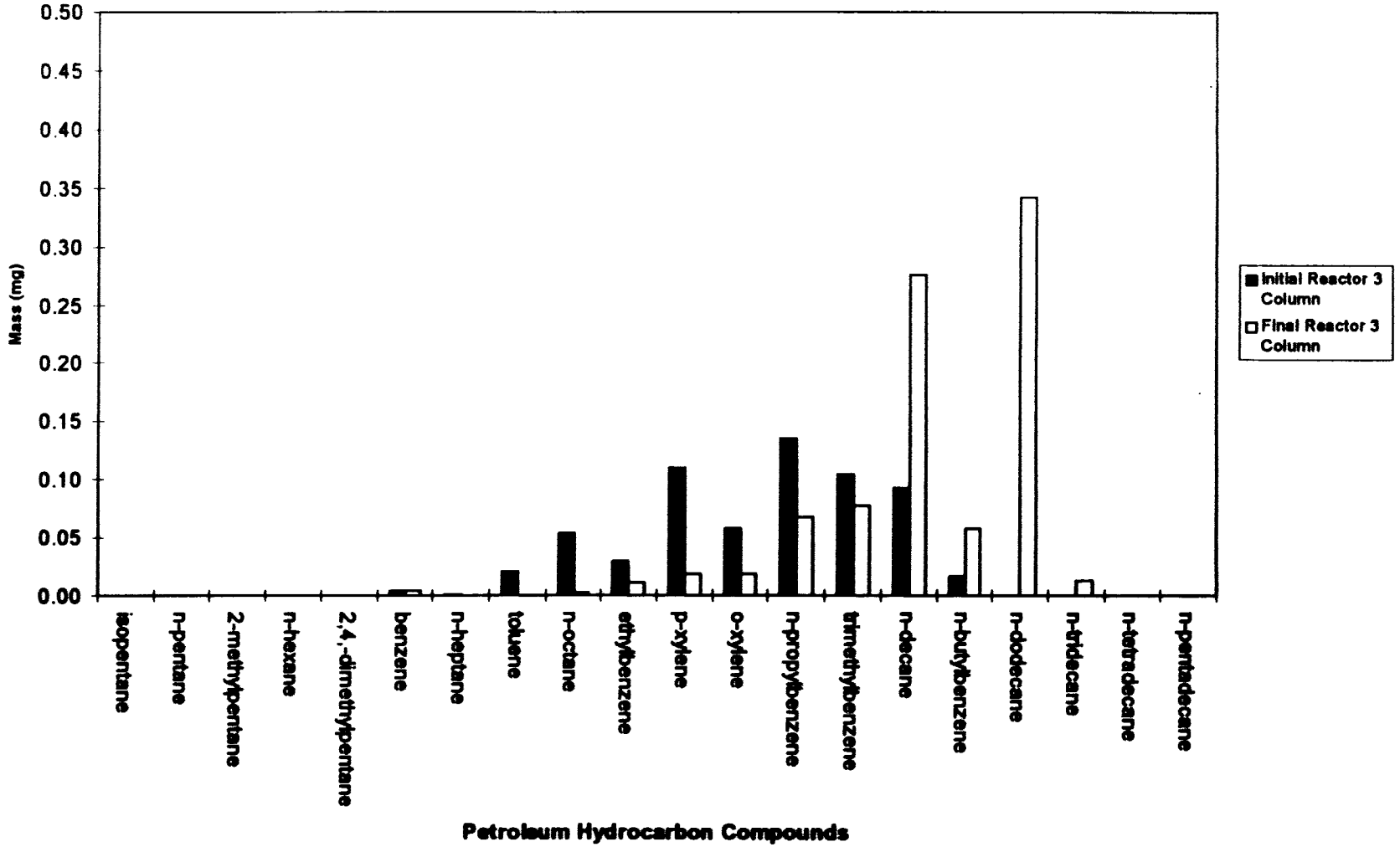

Figure 55. Initial and Average Final Mass of Petroleum Hydrocarbon Compounds (mg) in Column Reactors Containing Contaminated Soil with Microbial Amendment During Experiment #3.

Reactor 1, contaminated soil only, contained six petroleum hydrocarbon compounds at the start of the experiment (see Figure 54). The mass of *o*-xylene, *n*-propylbenzene, trimethylbenzene, and *n*-decane was greater at the termination of the experiment. The *n*-tridecane was completely consumed. Benzene, ethylbenzene, *p*-xylene, and *n*-pentadecane were present at the completion of the experiment. This condition contained the greatest amount of *n*-dodecane and *n*-tridecane.

The contaminated soil with the commercial microbial amendment (Reactor 3) contained more of the 20 compounds than the other two conditions, but in lower mass quantities. The TPH mass was comparable to that of the other conditions (see Table 21 in Section 6.3.3.2). Toluene and *n*-octane were consumed in the course of the experiment. The *n*-dodecane and *n*-pentadecane were present in the final analyses.

6.3.4 Soil Moisture

6.3.4.1 Column Reactors. The initial and final soil moisture contents for the column reactors used in Experiment #3 are presented in Table 22. The negative value for the percent average change indicates a net loss of soil moisture by the end of the 30-day incubation. Moisture loss occurred in each of the three experimental conditions; however, the loss was much less than the loss seen in Experiment #2. The reduced moisture loss may have been due to the new reactor arrangement. In this experiment, the column reactors were placed on their sides within an incubator, as explained in Section 6.1.2. The previous disappearances of moisture may have been due partially to the gravimetric drain of water from the top of the column reactor toward the bottom, because the column was mounted in an upright position. All moisture samples were taken within the top 2.5 to 3.0 inches of the soil column, possibly resulting in a false-negative moisture content. The soil moisture results for each of the three experimental conditions in Experiment #3 are more representative of the loss that might occur from evaporation during atmospheric exchanges.

If the column reactors are to remain in an upright position during the course of incubation, an additional homogenization step would need to be added to ensure that an accurate soil moisture value would be determined. However, the danger of homogenization after incubation is that experimental error may be introduced into the TPH and TOC analyses due to potential volatilization of TPH from the incubated soil.

Additionally, the lack of any values that indicate a net moisture addition in this experiment confirmed the water interference hypothesis previously discussed, and supported the decision to remove the water circulation system as the means for controlling temperature.

Table 22. Results of Soil Moisture Analysis (%) Conducted on Soil Samples from Column Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	19.45	18.60	19.29	19.21	
2	20.32	19.70	18.10	18.47	
3	20.17	21.81	20.22	18.62	-3.23
Average	19.98	20.04	19.20	18.77	
Contaminated Soil with Sterilized Amendment Addition					
1	19.80	18.83	19.25	18.97	
2	19.68	18.17	18.92	18.51	
3	19.84	18.81	18.34	18.51	-5.44
Average	19.78	18.60	18.84	18.66	
Contaminated Soil with Amendment Addition					
1	18.80	18.23	18.51	18.92	
2	19.13	18.23	18.04	18.63	
3	19.84	17.43	17.44	18.79	-5.23
Average	19.25	17.96	18.00	18.78	

6.3.4.2 Biometer Flask Reactors. The initial and final soil moisture values for the biometer flask reactors used in Experiment #3 are presented in Table 23. The values for each of the three experimental conditions are very similar to the values reported for the column reactors in this experiment. The average change from initial to final soil moisture values for each condition was minimal. Therefore, the biometer flask reactor design had a similar efficiency for minimizing the loss of moisture from the system.

6.3.5 Soil pH

6.3.5.1 Column Reactors. The initial and final soil pH values for the column reactors in Experiment 3 are presented in Table 24. The contaminated control was the only condition to exhibit an acidic change, although it was minimal. The remaining two conditions (contaminated soil with sterilized amendment and contaminated soil with addition of JP-4 and amendment condition) each indicated a slight increase in pH by the end of the incubation period. The condition with sterilized amendment resulted in a pH increase almost twice the increase indicated in the condition with added amendment and JP-4 jet fuel.

6.3.5.2 Biometer Flask Reactors. The initial and final soil pH values for the biometer flask reactors in Experiment #3 are presented in Table 25. The values reported in Table 25 followed the same trend that resulted from the column reactor analyses presented in Table 24. The pH value for the contaminated control decreased slightly, whereas the remaining two experimental conditions indicated a slight increase in pH. Additionally, the contaminated soil and sterilized amendment condition nearly doubled the pH change indicated for the contaminated soil with amendment condition, as had been indicated for this condition using the column reactor design. This mimicking of the trend between the two different reactor types further supports the usefulness of a flask setup as an alternative reactor design.

Table 23. Results of Soil Moisture Analysis (%) Conducted on Soil Samples from Biometer Flask Reactors During Experiment #3.

Contaminated Soil with No Amendment					
Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
1	19.45	18.64	NS	18.89	
2	20.32	18.53	NS	17.34	
3	20.17	18.06	19.10	17.86	
Average	19.98	18.41	19.10	18.03	-7.34
Contaminated Soil with Sterilized Amendment Addition					
Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
1	19.80	18.55	18.35	18.99	
2	19.68	19.63	18.31	19.02	
3	19.84	19.96	19.87	18.63	
Average	19.78	19.38	18.84	18.88	-3.75
Contaminated Soil with Amendment Addition					
Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
1	18.80	18.91	17.61	18.63	
2	19.13	18.16	17.56	18.04	
3	19.84	17.58	18.75	18.20	
Average	19.25	18.22	17.97	18.29	-5.67

Table 24. Results from pH Analysis Conducted on Soil Samples from Column Reactors During Experiment #3.

Conditions	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Final Average	pH Change
Contaminated Soil with No Amendment	6.60	6.40	6.40	6.40	6.40	-0.20
Contaminated Soil with Sterilized Amendment Addition	6.30	7.00	6.90	7.10	7.00	0.70
Contaminated Soil with Amendment Addition	6.90	7.20	7.30	7.30	7.27	0.37

Table 25. Results from pH Analysis Conducted on Soil Samples from Biometer Flask Reactors During Experiment #3.

Conditions	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Final Average	pH Change
Contaminated Soil with No Amendment	6.60	6.50	6.70	6.50	6.57	-0.03
Contaminated Soil with Sterilized Amendment Addition	6.30	7.30	7.30	7.20	7.25	0.95
Contaminated Soil with Amendment Addition	6.90	7.40	7.40	7.40	7.40	0.50

6.3.6 Dehydrogenase Activity in Soil Samples

6.3.6.1 Column Reactors. The results for dehydrogenase activity in the column reactors for Experiment #3 are presented in Table 26. The values depicted in the table indicate a net positive increase in dehydrogenase activity over the incubation period for all three experimental conditions. The lowest average change appears in the contaminated control reactors where a value of 10.3% was reported. The experimental condition with commercial amendment resulted in an average change over incubation of approximately 26%. The greatest change over the course of the incubation occurred in the experimental condition with sterilized amendment, which resulted in an average change of approximately 227%.

The relativity of the reported values per experimental condition was expected. The control condition was expected to have the least change in dehydrogenase activity by the end of the incubation period, whereas an effective amendment addition was expected to result in a substantial increase in dehydrogenase activity.

The results expressed in Table 26 suggest that the increased assay incubation time may have had a positive effect on the enhancement of TPF detection. However, although the values between experimental conditions differed greatly, the differences between the initial and final values within experimental conditions remained minimal. If dehydrogenase activity determinations are to be used to draw a relative comparison among the different experimental conditions, then the differences among the conditions should vary by magnitude so that a qualitative comparison can be made with certainty.

The incubation times for the dehydrogenase activity assays were increased from 48 hours to 1 week. This increased incubation time resulted in the enhanced TPF detection explained previously. The added benefit of enhanced detection observed by increasing the incubation time for an additional 5 days was minimal. It is not known whether increasing the assay incubation beyond 1 week would further enhance TPF detection. However, it seems that a further lengthening of incubation time would only delay interpretation of the experimental results.

Table 26. Results of Dehydrogenase Activity Analysis (µg-H/g-dry soil) Conducted on Soil Samples from Column Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	1.23e+03	8.76e+02	5.49e+02	7.79e+02	
2	5.90e+02	1.29e+03	6.83e+02	6.58e+02	
3	4.83e+02	8.75e+02	8.90e+02	1.02e+03	
Average	7.68e+02	1.01e+03	7.07e+02	8.19e+02	10.29
Contaminated Soil with Sterilized Amendment Addition					
1	8.69e+03	3.26e+04	2.96e+04	2.31e+04	
2	9.40e+03	3.34e+04	3.59e+04	2.24e+04	
3	7.98e+03	4.49e+04	1.60e+04	1.76e+04	
Average	8.69e+03	3.70e+04	2.72e+04	2.10e+04	226.68
Contaminated Soil with Amendment Addition					
1	1.23e+04	9.36e+03	1.44e+04	1.26e+04	
2	1.09e+04	1.01e+04	9.58e+03	1.45e+04	
3	6.14e+03	1.37e+04	1.19e+04	1.44e+04	
Average	9.78e+03	1.11e+04	1.20e+04	1.38e+04	25.59

Table 27. Results from Dehydrogenase Activity Analysis (µg-H/g-dry soil) Conducted on Soil Samples from Biometer Flask Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	1.23e+03	1.60e+03	4.96e+02	9.68e+02	
2	5.90e+02	1.16e+03	7.86e+02	8.26e+02	
3	4.83e+02	3.10e+02	4.03e+02	5.55e+02	
Average	7.68e+02	1.02e+03	5.62e+02	7.83e+02	2.82
Contaminated Soil with Sterilized Amendment Addition					
1	8.69e+03	3.46e+04	2.57e+04	2.76e+04	
2	9.40e+03	3.13e+04	3.19e+04	2.90e+04	
3	7.98e+03	2.31e+04	3.76e+04	3.78e+04	
Average	8.69e+03	2.97e+04	3.17e+04	3.15e+04	256.22
Contaminated Soil with Amendment Addition					
1	1.23e+04	1.17e+04	1.55e+04	1.09e+04	
2	1.09e+04	1.52e+04	9.27e+03	1.60e+04	
3	6.14e+03	1.62e+04	1.71e+04	1.20e+04	
Average	9.78e+03	1.44e+04	1.40e+04	1.30e+04	40.73

Table 28. Results from Total Plate Count Microbial Enumeration Analysis (CFU/g-dry soil) Conducted on Soil Samples from Column Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	7.78×10^2	5.85×10^7	3.89×10^7	2.11×10^7	
2	8.30×10^2	3.53×10^7	4.30×10^7	2.04×10^7	
3	7.63×10^2	4.16×10^7	3.81×10^7	2.32×10^7	
Average	7.90×10^2	4.51×10^7	4.00×10^7	2.16×10^7	4492104
Contaminated Soil with Sterilized Amendment Addition					
1	4.94×10^2	6.42×10^5	8.00×10^5	2.83×10^6	
2	4.49×10^2	7.68×10^5	6.27×10^5	2.19×10^6	
3	5.07×10^2	6.75×10^5	5.85×10^5	9.86×10^5	
Average	4.83×10^2	6.95×10^5	6.71×10^5	2.00×10^6	141308
Contaminated Soil with Amendment Addition					
1	1.14×10^6	2.31×10^6	3.95×10^6	1.36×10^6	
2	6.69×10^5	2.56×10^6	3.77×10^6	1.94×10^6	
3	8.80×10^5	4.04×10^6	4.46×10^6	2.52×10^6	
Average	8.95×10^5	2.97×10^6	4.06×10^6	1.94×10^6	234.08

for biometer flask reactors in Experiment #3 are presented in Table 32, Section 6.3.7.2. The results for the two reactor configurations are very similar following the same general trend.

The results indicate that approximately 3.8% of the heterotrophic increase in column-reactors for the contaminated control presented in Table 31, is accounted for by JP-4 degrading bacteria. The value determined for the biometer flask configuration is approximately 2.6%. These microbial increases occurred without the introduction of any nutrients or bacteria. Furthermore, this increase is substantial compared to the increase in JP-4 degrading bacteria in the two experimental conditions.

The results presented here may depict a situation in which the nutrient portion of the amendment addition stimulated indigenous bacterial or protozoan growth. If this were to occur, the indigenous organisms might consume components from the inoculum as a carbon and/or energy source to support further growth, this consumption could include bacteria from the inoculum. However, it is also possible that rapid depletion of JP-4 initiated the decay and eventual death phase of the population in the two experimental conditions. However, it is difficult to draw such conclusions without further data. The data may again give a misrepresentation of the effect of nutrient and amendment addition on enhancing biodegradation of JP-4 jet fuel, because of the "incubation window" in which the samples were taken.

Table 29. Results from JP-4 Degrader Microbial Enumeration Analysis (CFU/g-dry soil) Conducted on Soil Samples from Column Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	1.11×10^4	1.08×10^6	1.50×10^6	1.97×10^6	
2	1.20×10^4	1.12×10^6	9.23×10^5	1.77×10^6	
3	1.31×10^4	1.32×10^6	6.44×10^5	1.79×10^6	
Average	1.21×10^4	1.17×10^6	1.02×10^6	1.84×10^6	11001
Contaminated Soil with Sterilized Amendment Addition					
1	1.03×10^4	6.25×10^4	5.65×10^4	7.70×10^4	
2	1.33×10^4	5.76×10^4	3.94×10^4	9.11×10^4	
3	9.96×10^3	5.33×10^4	4.75×10^4	4.13×10^4	
Average	1.12×10^4	5.78×10^4	4.78×10^4	6.98×10^4	371.43
Contaminated Soil with Amendment Addition					
1	3.71×10^4	5.03×10^6	1.98×10^6	1.22×10^5	
2	3.15×10^4	3.90×10^6	1.17×10^6	2.75×10^5	
3	3.29×10^4	3.45×10^6	9.22×10^5	2.35×10^5	
Average	3.38×10^4	4.13×10^6	1.36×10^6	2.11×10^5	5522

Table 30. Results from Mid Point Microbial Enumeration Analysis (CFU/g-dry soil) Conducted on Soil Samples from Column Reactors During Experiment #3.

Contaminated Soil with No Amendment		
Replicate	Total Count	BIM with JP-4
1	8.20×10^6	6.68×10^6
2	7.08×10^6	6.12×10^7
3	1.06×10^7	9.93×10^6
Average	8.63×10^6	2.59×10^7
Contaminated Soil with Sterilized Amendment Addition		
Replicate	Total Count	BIM with JP-4
1	1.13×10^8	8.47×10^7
2	1.35×10^8	1.15×10^8
3	1.20×10^8	9.81×10^8
Average	1.23×10^8	9.81×10^7
Contaminated Soil with Amendment Addition		
Replicate	Total Count	BIM with JP-4
1	1.22×10^8	1.28×10^8
2	7.37×10^7	6.90×10^7
3	1.26×10^8	1.62×10^8
Average	1.07×10^8	1.20×10^8

6.3.7.2 Biometer Flask Reactors. Table 31 depicts the initial and final values determined for viable heterotrophs in biometer flask reactors for Experiment #3. The positive values represented for the average change indicate an increase in the microbial population by the end of the 30-day incubation period. The heterotrophic enumerations presented here are very similar to the values presented in Table 28 for the initial and final heterotrophic enumerations of the column reactors. The heterotrophic enumerations conducted for the biometer flask reactors indicated that there was a significant increase in heterotrophic bacteria for both the contaminated control and the experimental condition with added nutrients, and a smaller positive change for the experimental condition with added amendment.

As the results in Table 28 have suggested for the column reactor design, there does not appear to be any significant contribution of viable bacteria made by the nutrient or amendment addition over the contaminated control. In addition, there appeared to be a similar trend in the reduction of viable bacteria beyond the mid-incubation enumeration, as occurred in the column reactors for this experiment.

The data tabulated thus far for enumeration of heterotrophic and JP-4 degrading bacteria suggest that it is difficult to draw a comparative conclusion for relative counts between the contaminated control and the experimental conditions. Although the microbial enumeration data presented in this experiment seem to suggest that microbiological amendment or nutrient addition may not significantly increase the quantity of soil bacteria or affect growth, such a conclusion can not be confirmed by drawing one or two grab-samples over a 30-day incubation period. A one- or two-sample enumeration will not give an accurate representation of the bacterial growth cycle which is on going over the course of the soil incubation. To determine relative growth over the incubation period, a growth curve must be constructed comparing the different experimental conditions. This would involve a daily sampling and soil enumeration procedure, a task which is impractical for the purpose of this protocol. Because the enumerative data accumulated thus far indicate the existence of this problem, it may be suggested that the initial and final sampling for enumerative purposes be eliminated from the final protocol development. The microbial enumerations procedure would still include the enumerations for viable JP-4 degrading bacteria in the microbiological amendment, so that an accurate inoculum concentration could be determined.

The results for total viable heterotrophs and viable JP-4 jet fuel degraders determined for biometer flask reactors in Experiment #3 are presented in Table 31. The mid-incubation values for biometer flask reactors presented in Table 33 are very similar to the enumeration values presented in Table 30 for the column reactors. This consistency in results between the two reactor types further supports the ability to interchange the reactor design if needed.

Table 31. Results from Total Plate Count Microbial Enumeration Analysis (CFU/g-dry soil) Conducted on Soil Samples from Biometer Flask Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	7.78×10^2	3.35×10^7	3.48×10^7	4.64×10^7	
2	8.30×10^2	3.86×10^7	4.10×10^7	3.70×10^7	
3	7.63×10^2	4.95×10^7	4.61×10^7	3.97×10^7	
Average	7.91×10^2	4.05×10^7	4.06×10^7	4.10×10^7	5149499
Contaminated Soil with Sterilized Amendment Addition					
1	4.94×10^2	TNTC	6.31×10^5	9.51×10^5	
2	4.49×10^2	1.11×10^6	8.29×10^5	1.10×10^6	
3	5.07×10^2	TNTC	2.39×10^6	1.11×10^6	
Average	4.83×10^2	1.11×10^6	1.28×10^6	1.05×10^6	247312
Contaminated Soil with Amendment Addition					
1	1.14×10^6	TNTC	6.26×10^6	6.71×10^4	
2	6.69×10^5	3.69×10^6	3.08×10^6	5.09×10^4	
3	8.80×10^5	2.25×10^6	4.51×10^6	5.79×10^4	
Average	8.95×10^5	2.97×10^6	4.62×10^6	5.86×10^4	184.86

Table 32. Results from JP-4 Degrader Microbial Enumeration Analysis (CFU/g-dry soil) Conducted on Soil Samples from Biometer Flask Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	1.11×10^4	1.04×10^6	6.03×10^5	8.19×10^5	
2	1.20×10^4	9.44×10^5	1.58×10^6	6.17×10^5	
3	1.31×10^4	1.26×10^6	2.06×10^6	4.92×10^5	
Average	1.21×10^4	1.08×10^6	1.41×10^6	6.42×10^5	8528.10
Contaminated Soil with Sterilized Amendment Addition					
1	1.03×10^4	6.23×10^4	3.40×10^4	4.26×10^4	
2	1.33×10^4	4.02×10^4	TFTC	8.60×10^4	
3	9.96×10^3	TFTC	TFTC	1.36×10^5	
Average	1.12×10^4	5.13×10^4	3.40×10^4	8.82×10^4	280.80
Contaminated Soil with Amendment Addition					
1	3.71×10^4	1.14×10^6	7.68×10^5	1.39×10^6	
2	3.15×10^4	1.32×10^6	1.57×10^6	2.24×10^5	
3	3.29×10^4	7.02×10^5	8.23×10^5	6.03×10^5	
Average	3.38×10^4	1.06×10^6	1.05×10^6	7.40×10^5	2710.65

The contaminated control indicated a slight increase in JP-4 degrading bacteria over total heterotrophs, as indicated in Table 32 for the biometer flask reactor design. Again, this increase in JP-4 degraders most likely represents experimental error due to the variation among replicate samples.

The two experimental conditions indicate that the JP-4 degrading bacteria make up approximately 70 to 82% percent of the heterotrophic population. However, these values do not differ significantly from the control values and indicate that by mid-incubation there does not seem to be a significant contribution to the bacterial population by addition of nutrients or full amendment.

The microbial enumeration data from previous experiments suggested that by the end of the incubations there had been a net reduction in the amount of viable JP-4 degrading and heterotrophic bacteria. It was for this reason that microbial enumerations were conducted at a point midway through the 30-day incubation. Previous data had not suggested that much growth phase had occurred. The inability to detect microbial growth was most likely due to the "window" in which the microorganisms were enumerated. Waiting to take final samples for enumeration purposes at the end of the incubation period most likely allowed for the introduction of early cell death phase. enumerations made at mid-incubation allowed for a more accurate interpretation of the microbial growth occurring in the reactor by retrieving samples before death phase occurred.

Bacterial enumerations at the midpoint of the incubation period (15 days) for Experiment #3 were determined for total viable heterotrophs and for viable JP-4 jet fuel degraders. The results of these enumerations are presented in Table 33. The results for the enumerations indicate that in the contaminated control bacteria capable of degrading JP-4 jet fuel slightly outnumbered total heterotrophic bacteria. This event is unlikely because JP-4 degraders make up a fraction of the viable heterotrophic bacteria and generally should not exceed the heterotrophic count. However, it may be possible that a portion of the indigenous bacteria in the contaminated control had become specialized to degrade components of JP-4, and had lost the ability to degrade other organic carbon components common to most heterotrophs. This would account for the slight increase in viable JP-4 degraders over viable heterotrophic bacteria in the control. However, this is unlikely and the difference probably should be attributed to experimental error.

In the experimental condition with added nutrients, JP-4 jet fuel-degrading bacteria make up approximately 79% of the total bacterial count at the midpoint of incubation. In the experimental condition with amendment, the values for JP-4-degrading and heterotrophic bacteria are very similar, with the JP-4 degraders slightly greater in number.

In both experimental conditions, the values for viable JP-4-degrading bacteria and heterotrophic bacteria are extremely close, indicating that the results from the addition of the full amendment do not differ

significantly from the results incurred by adding the nutrients only. Furthermore, the results indicated in Table 33 for the two experimental conditions do not differ significantly from the values representing the contaminated control. This may indicate that the microbiological and nutrient addition during Experiment #3 had no significant impact in increasing viable bacteria capable of degrading JP-4 jet fuel.

The values represented in Table 33 indicate that by day 15 of the incubation the bacterial population had experienced a growth increase. JP-4-degrading and heterotrophic bacterial enumerations were made for the WMI 2000 inoculum prior to dosing (data not shown). The bacterial inoculum for JP-4 degraders and heterotrophic bacteria was determined to be 2.17×10^5 CFU/g-dry soil and 3.9×10^6 CFU/g-dry soil, respectively. The increase in values for both enumerations by mid-incubation indicate an increased microbial population. However, the increase encountered in the two experimental conditions does not greatly exceed the values which represent the contaminated control and may indicate that under these particular soil conditions (nutrient availability, etc.) that indigenous bacteria are sufficient for carrying out JP-4 degradation.

6.3.8 Nutrient Concentrations in Soil Samples

6.3.8.1 Column Reactors. The initial and final results for soil nutrients and cation exchange capacity for column reactor soils are presented in Table 34. The data obtained from this analysis gave valuable insight into the nutrient dosing. The nutrient dosage for Experiment #3 was 100 times greater than the nutrient dosage for Experiments #1 and #2.

The higher loading in this experiment resulted in increased initial concentrations of nitrogen (N), phosphorus (P), potassium (K), and sodium (Na) in both of the experimental conditions. There did not appear to be any increase in the concentrations of calcium (Ca) and magnesium (Mg), except in one case where the initial Ca concentration exceeded the background concentration by approximately 160 ppm. This occurred in the experimental condition with added amendment.

Table 33. Results of Mid Point Microbial Enumeration Analysis (CFU/g-dry soil) Conducted on Soil Samples from Biometer Flask Reactors During Experiment #3.

Contaminated Soil with No Amendment		
Replicate	Total Count	BIM with JP-4
1	9.05×10^6	9.65×10^6
2	7.24×10^6	9.08×10^6
3	1.03×10^7	1.05×10^7
Average	8.87×10^6	9.73×10^6
Contaminated Soil with Sterilized Amendment Addition		
Replicate	Total Count	BIM with JP-4
1	1.99×10^7	1.70×10^7
2	3.85×10^6	1.18×10^6
3	2.84×10^7	2.51×10^7
Average	1.74×10^7	1.44×10^7
Contaminated Soil with Amendment Addition		
Replicate	Total Count	BIM with JP-4
1	2.50×10^7	3.00×10^7
2	7.25×10^7	2.60×10^7
3	1.44×10^7	2.24×10^7
Average	3.73×10^7	2.62×10^7

Table 34. Results of Cation Exchange Capacity and Nutrient Analysis Conducted on Soil Samples from Column Reactors During Experiment #3.

	CEC (meq/100 g)	N (ppm)	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	Na (ppm)
Contaminated Soil with No Amendment							
Final Reactor A	2.5	400	42	24	400	22	11
Final Reactor B	2.5	780	9	23	400	23	7
Final Reactor C	2.4	500	12	20	380	23	11
Average	2.5	560	21	22	393	23	10
Average Change (%)	12	-28	91	12	9	13	61
Contaminated Soil with Sterilized Amendment Addition							
Initial	2.8	1020	126	43	370	30	76
Final Reactor A	1.9	620	120	27	270	19	63
Final Reactor B	2.3	620	133	37	320	22	78
Final Reactor C	2.4	610	118	37	340	24	83
Average	2	615	126	37	330	23	81
Average Change (%)	-16	-40	0	-14	-11	-23	6
Contaminated Soil with Amendment Addition							
Initial	3.3	1160	116	35	530	27	69
Final Reactor A	3.3	400	112	40	510	33	79
Final Reactor B	3.1	620	122	35	490	30	77
Final Reactor C	3.1	590	112	34	480	30	76
Average	3	537	115	36	493	31	77
Average Change (%)	-4	-54	-1	4	-7	15	12

The data indicated that there were sufficient amounts of these key nutrients in the soil by the end of the incubation period. Considerable nitrogen usage was reported in the two experimental conditions, as well as in the contaminated control. Nitrogen was utilized more readily than any of the other soil nutrients, and its disappearance was greater in the two experimental conditions.

The nutrient concentration analyses typically are conducted using sample sizes in excess of 100 g. However, due to the reactor sizes used in these experiments, it was difficult to meet this requirement. Therefore, the nutrient concentration analyses conducted by A&L Laboratories, required special handling and an associated additional charge for each sample. Because the key nutrients of interest appear to be nitrogen and phosphorus, it may be more cost effective to analyze for these nutrients only.

6.3.8.2 Biometer Flask Reactors. The results for the initial and final nutrient analyses and cation exchange capacities conducted for the biometer flask reactors in Experiment #3 are presented in Table 35. The results are very similar to the results presented in Table 34 for the column reactor design used during this experiment. Increases above background concentrations occurred for nitrogen (N), phosphorus (P), potassium (K), and sodium (Na) in both of the experimental conditions. The nutrient dosing in this experiment resulted in an initial excess of these elements above background levels. The values depicted for nitrogen in Table 35 indicate that once again nitrogen was an essential element for microbiological growth. The final values reported in this table indicated that there was significant disappearance of nitrogen over the course of the incubation and that the other nutrients were removed to a lesser extent, if at all.

Because the results presented in Table 35 are consistent with the results presented in Table 34 for the column reactor design, it appears that the flask setup could serve as a viable alternative for a reactor design. Additionally, increasing the size of the flask reactor would allow for more sample to meet the analytical requirements for specific analyses, such as the nutrient concentration analyses conducted by A&L Laboratories. A wide-mouth flask design would facilitate the sampling procedure and ensure collection of a homogeneous sample.

6.3.9 Carbon in Soil Samples

6.3.9.1 Column Reactors. The results of the initial and final inorganic carbon analyses conducted for column reactors in Experiment #3 are presented in Table 36. The procedure for these analyses was altered in order to facilitate the analytical process. All of the samples were processed at 1,000°C to obtain the

Table 35. Results of Cation Exchange Capacity and Nutrient Analysis Conducted on Soil Samples from Biometer Flask Reactors During Experiment #3.

ID	CEC (meq/100 g)	N (ppm)	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	Na (ppm)
Contaminated Soil with No Amendment							
Final Reactor A	2.7	300	20	31	420	26	13
Final Reactor B	2.3	620	14	29	370	24	14
Final Reactor C	3.4	560	10	80	440	84	19
Average	2.5	493	15	47	410	45	15
Average Change (%)	12	-37	33	133	14	123	156
Contaminated Soil with Sterilized Amendment Addition							
Initial	2.8	1020	126	43	370	30	76
Final Reactor A	2.3	700	118	52	320	25	74
Final Reactor B	2.3	700	116	45	330	27	78
Final Reactor C	2.5	500	121	50	360	28	88
Average	2	600	119	48	345	28	83
Average Change (%)	-14	-41	-6	10	-7	-8	9
Contaminated Soil with Amendment Addition							
Initial	3.3	1160	116	35	530	27	69
Final Reactor A	3.0	620	117	44	460	32	663
Final Reactor B	3.5	600	117	58	540	36	80
Final Reactor C	2.7	900	111	46	420	24	62
Average	3	707	115	49	473	31	268
Average Change (%)	-7	-39	-1	41	-11	14	289

Table 36. Results of Inorganic Carbon Analysis (mg-C/Kg-dry soil) Conducted Using the UIC Method on Soil Samples from Column Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	398	292	6904	1533	
2	349	3019	656	-381	
3	423	467	477	856	
Average	390	1259	2679	669	293.57
Contaminated Soil with Sterilized Amendment Addition					
1	641	1326	35	275	
2	408	1364	375	608	
3	301	744	730	729	
Average	450	1145	380	537	52.75
Contaminated Soil with Amendment Addition					
1	879	-720	780	-867	
2	830	-2315	256	-379	
3	691	147	449	263	
Average	800	-963	495	-328	-133.15

total carbon value for each sample. After all the total carbon analyses were completed, the sample boats were cleaned and loaded with fresh sample. These samples were combusted at 550°C to obtain the organic matter content values. The inorganic fraction was calculated as the difference of the total carbon and organic carbon. Although this method greatly facilitated the analytical process, the variability between reactor replicates skewed the data enough to cause a calculational error. This error resulted in a negative inorganic carbon value. The negative values depicted in Table 36 indicate that the total carbon values were less than the organic carbon fraction. This is impossible, because the organic carbon is a fraction of the total carbon. Thus, the heterogeneity of the sample again played a role in sample deviation, and inorganic analyses resulted in erroneous and unusable data.

Table 37 depicts the resulting values for organic carbon analyses conducted for column reactors in Experiment #3 using the UIC method previously described. The values produced using the combustion at 550°C represented the organic carbon matter in the soil and are presented in Table 38. The variability among reactor replicates was slight in comparison to previously presented carbon data. As a result, the data presented in Table 38 depict a more realistic percent change for organic carbon over the 30-day incubation period. The contaminated control indicated the least change with a slight removal of organic carbon, whereas the two experimental conditions indicated a greater organic carbon removal with changes in values of approximately 12.8%.

The organic matter values depicted in Table 38 are a result of employing the muffle method for analysis. These values, which are approximately two times greater than the values presented in Table 37, have increased variability between replicates, resulting in erroneous percent changes for both the experimental conditions and the contaminated control. Even though a more representative sample is analyzed by the muffle method, a modification to the procedure must be made in order to increase the sample homogeneity and to obtain meaningful results.

6.3.9.2 **Biometer Flask Reactors.** Table 39 presents the results obtained from the analysis for inorganic carbon in biometer flask reactor soils in Experiment #3. Although the method was modified, several erroneous inorganic carbon values resulted. The resulting errors made it impossible to make any comparisons between the experimental conditions.

Table 37. Results of Organic Carbon Analysis (mg-C/Kg-dry soil) Conducted Using the UIC Method on Soil Samples from Column Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	8204	7246	7729	7451	
2	7390	6758	6684	7696	
3	6797	7262	6872	7856	
Average	7464	7089	7095	7668	-2.41
Contaminated Soil with Sterilized Amendment Addition					
1	8410	7478	7166	7411	
2	7488	6436	6600	7083	
3	7875	6767	6757	6495	
Average	7924	6894	6841	6996	-12.80
Contaminated Soil with Amendment Addition					
1	8776	8310	7487	8686	
2	9756	9716	7605	7321	
3	8561	7704	7038	6936	
Average	9031	8577	7377	7648	-12.89

Table 38. Initial and Final Organic Carbon (mg-C/Kg dry soil) Results for Column-Reactors in Experiment 3, Using the Muffle Furnace Method for Analyses.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	12898	13285	12719	12535	
2	13085	13453	12986	13456	
3	13019	24674	13790	14140	
Average	13001	17137	13165	13377	11.99
Contaminated Soil with Sterilized Amendment Addition					
1	14184	12068	13155	13240	
2	14349	28238	14336	12551	
3	14609	12352	12962	13130	
Average	14381	17553	13484	12974	2.01
Contaminated Soil with Amendment Addition					
1	14668	13199	13527	17369	
2	15014	13543	13007	13223	
3	15648	13825	12825	12582	
Average	15110	13522	13120	14391	-9.48

The results of the initial and final organic carbon analyses determined using the UIC method are depicted in Table 40. The values presented in this table indicated very slight deviation among reactor replicate samples. Thus, the average change among the two experimental conditions and the contaminated control was calculated with greater certainty.

The comparative organic carbon values obtained by muffling are presented in Table 41. The results presented in this table are similar to the organic carbon results obtained for the column reactors in this experiment. The variability between replicate samples of reactors made it difficult to reach any relative conclusions between the two experimental conditions and the contaminated control.

6.3.10 Particle Size Distribution

6.3.10.1 Column Reactors. Soil particle size distribution and classification analyses were not conducted for column reactors in Experiment #3. It was expected that these soil parameters would remain consistent with the results in Experiment #1, because the same soil-type was used.

6.3.10.2 Biometer Flask Reactors. Soil particle size distribution and textural classification analyses were not conducted for the biometer flask reactors in Experiment #3. It was expected that these soil parameters would remain consistent with the results in Experiment #1, because the same soil type was used.

6.3.11 Cation Exchange Capacity

6.3.11.1 Column Reactors. Cation exchange capacity analyses were not conducted for the column reactors in Experiment #3, as explained previously in Section 6.2.1. Because the same soil was used for all three experiments, it was assumed that the initial CEC values for the soil would not deviate from the values reported for the soil in Experiment #1.

6.3.11.2 Biometer Flask Reactors. Cation exchange capacity analyses were not conducted for the biometer flask reactors in Experiment #3. As mentioned above, it was assumed that the initial CEC values for the soil would not deviate from the values reported for the soil in Experiment #1.

Table 39. Results from Inorganic Carbon Analysis (mg-C/Kg-dry soil) Conducted Using the UIC Method on Soil Samples from Biometer Flask Reactors During Experiment #3.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	398	-464	1288	406	
2	349	-1593	4403	407	
3	423	814	272	724	
Average	390	-414	1988	512	78.12
Contaminated Soil with Sterilized Amendment Addition					
1	641	2160	551	-550	
2	408	244	196	-1172	
3	301	135	-2729	688	
Average	450	846	-661	-345	-111.81
Contaminated Soil with Amendment Addition					
1	879	-789	-4901	661	
2	830	3385	1592	169	
3	691	-224	1453	13	
Average	800	790	-619	281	-81.14

Table 40. Initial and Final Organic Carbon (mg-C/Kg-dry soil) Results for Biometer Flask-Reactors in Experiment 3, Using the UIC Method for Analysis.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	8204	8228	7224	8833	
2	7390	9374	7467	7616	
3	6797	7981	7579	7553	
Average	7464	8528	7423	8000	6.97
Contaminated Soil with Sterilized Amendment Addition					
1	8410	6955	7022	7682	
2	7488	7502	7338	8007	
3	7875	7543	10376	6191	
Average	7924	7333	8245	7294	-3.79
Contaminated Soil with Amendment Addition					
1	8776	7475	11565	7192	
2	9756	7063	5821	7014	
3	8561	7010	5537	7415	
Average	9031	7183	7641	7207	-18.69

Table 41. Initial and Final Organic Carbon (mg-C/Kg-dry soil) Results for Biometer Flask-Reactors in Experiment 3, Using the Muffle Furnace Method for Analysis.

Replicate	Initial	Final Reactor A	Final Reactor B	Final Reactor C	Average Change (%)
Contaminated Soil with No Amendment					
1	12898	14277	12837	12599	
2	13085	13174	13702	13102	
3	13019	14518	12845	13003	
Average	13001	13990	13128	12901	2.61
Contaminated Soil with Sterilized Amendment Addition					
1	14184	12909	12672	12807	
2	14349	13406	13328	12934	
3	14609	5729	12972	14683	
Average	14381	10681	12991	13475	-13.90
Contaminated Soil with Amendment Addition					
1	14668	12850	13062	13755	
2	15014	13116	13045	13987	
3	15648	12614	35574	13036	
Average	15110	12860	20560	13593	3.71

7.0 DISCUSSION

The purpose of conducting the experiments described in this report was to examine various reactor configurations, experimental procedures, and analytical methods to develop a method to reliably evaluate commercially available microbiological amendment products. It is noted that the experiments conducted during the development of the protocol were not conducted to evaluate the WMI-2000 product supplied by WMI Inc., of Houston Texas. This company provided Battelle with some of their product free of charge as they were interested in the testing and they had faith in their product. It is suggested that the WMI-2000 product be submitted for evaluation under the finalized protocol. The changes made in inoculation procedures between experiments were not directed by the manufacturer and were not intended to have any implications on the amendment performance. The changes were made to allow better evaluation and screening of the protocol methods.

The methods used in the first experiment followed the methods described in the Experimental Design Test Plan (Battelle, 1994). The experimental methods in the plan include standard laboratory procedures for conducting experiments to examine contaminant biodegradation. An excessive number of analytical protocols were included in the first experiment to allow investigations to determine which protocols provide the best data for evaluation purposes. The results from this experiment were used to select or eliminate, or to make modifications to the original methods in the protocol developmental process.

Although the results from the first experiment did not show significant differences between experimental conditions, there were several necessary modifications that became apparent and were incorporated into the second experiment. The amendment was adjusted so that the microorganisms in the inoculum were increased 100-fold so that any enhanced degradation or increased population would be more easily detected.

The first method modification was a shortening of the incubation period from 60 days to 30 days. This was necessary because the contaminant was reduced to a level in all conditions that made any comparison difficult. Because the purpose of the evaluation is to compare the amendment against indigenous activity, it is necessary to have contaminant remaining at the end of the test.

Slight modifications were made to the reactor setup and operation. Glass wool was placed in the columns to keep the soil off the end cap. Valve operation during atmospheric exchanging was optimized to ensure consistent flushing.

The analytical protocols for measuring soil pH, dehydrogenase activity, and microbial populations were slightly modified. The pH protocol was modified to allow a longer equilibration time for more accurate

analysis. The dehydrogenase activity assay was modified to increase the sensitivity and to reduce the amount of methanol waste produced. The microbial enumeration procedure was modified to decrease chemical usage, prevent cross-contamination, and increase staff efficiency.

The UIC method for carbon analysis did not provide good data, generated a significant amount of waste, and was extremely time consuming. The UIC method uses a very small sample and the heterogeneities in the soils cause a large variation in the data. Because of these reasons, an additional method was included in the second experiment for comparison purposes. The method was a standard method for gravimetrically measuring organic content in soils (Gardner, 1965).

The second experiment was plagued by leakage problems resulting in cooling water getting into the soil chamber and flooding the sample. Upon investigation, it was determined that the inner o-ring seals were torn by the compression of soil particles and the Teflon™ lip on the column end cap. It is difficult to monitor for internal leakage and this problem indicated that the use of the recirculating water was suitable for this experiment. The third experiment was conducted by placing the columns in a temperature-controlled incubator.

In addition to the leakage problem, there were a number of problems that became apparent with the use of the column reactors. First, the moisture in the column settled toward the bottom over the incubation period. To overcome this, the columns were placed on their sides in the incubator. Another problem was the inability to collect representative samples during reactor harvesting. The soils could not be homogenized to any extent in the columns and sampling was done from end to end. This was determined to have an undesirable impact on the analyses depending on where in the column the sample was pulled. Removing the soil from the reactor for harvesting would be inappropriate as a significant amount of the contaminant would be lost through adsorption to surfaces and through volatilization. Because these problems were encountered, biometer flask reactors were included in the third experiment for comparison purposes.

The atmosphere exchanging procedure used in the second experiment was further modified during the third experiment to both simplify the process and to ensure thorough flushing. The finalized procedure entailed direct flushing of 100 mL of air from a syringe through the reactor and into Tedlar™ gas sampling bags. This method assured that a 100-mL volume was passed through the reactor and collected and that the reactor was at ambient pressure following the flush.

The results from the second experiment indicated that the 30-day incubation period was appropriate and that the modifications to the pH and microbial enumeration protocols were beneficial. The pH procedure was modified to minimize the potential for evaporative losses and to facilitate easier electrode insertion. The results from the enumeration procedure indicated that the soil counts were less reliable than the counts made

on the inoculum. An intermediate enumeration was incorporated in the third experiment to determine if the lack of significant differences in populations was an artifact of time or if the recovery of cells was not effective. Total plate counts also were made during the third experiment to determine if the hydrocarbon degraders made up a significant proportion of the total microbial population and if this analysis was valuable for the protocol.

The dehydrogenase activity assay still was not sensitive enough to register significant activity. An attempt was made to increase the sensitivity of the assay for the third experiment by increasing the incubation period from 2 days to 5 days. Conducting the muffling procedure for the organic matter analysis proved more efficient than the UIC method, and the data for the organic content was more representative than the UIC method. This was attributed to the larger sample size being more representative of the soil as a whole. Both analytical methods were included in the third experiment to further evaluate their use in the final protocol.

The results from the third experiment were used to finalize the protocol. It was determined that the flask approach was superior to the column approach because of the ability to homogenize the soils in the flask prior to removal, the larger headspace that reduced the flushing requirements by providing more oxygen and reduced volatile losses, and the capacity to use more soil. It is recommended that a 500-mL flask equipped with a double syringe needle and valve gas exchange system be used in the testing protocol. One needle should be positioned into the soil and the other near the top of the flask. The gas can be exchanged using the direct-flush procedure described above. Using this configuration and method for flushing atmospheres would more closely simulate in situ venting than the biometer flask configuration and would save considerable cost over the use of columns or biometer flasks.

It was determined that the methods for nutrient analysis, soil characteristic analyses, contaminant analyses, and oxygen and carbon analyses were effective and appropriate for inclusion in the protocol. The results from the soil pH analyses conducted according to the test plan (Battelle, 1994) and during the nutrient analysis were comparable, so to avoid repetition, the pH determined during nutrient analyses is included in the protocol. Based on the results from the second and third experiments, it was determined that the gravimetric method of carbon analysis was better than the UIC method and should be included in the protocol. Alkalinity analysis can be included in the place of the UIC method for inorganic carbon analysis. Another benefit of using the gravimetric method for measuring organic content is that the procedure can be combined with the soil moisture analysis. The soil moisture analysis was modified so that, following the final weighing step, the material could be combusted and the organic matter analysis could be conducted. This modification requires the use of crucibles in place of aluminum weighing dishes and a larger sample volume for accurate weighing.

8.0 CONCLUSIONS

Three experimental runs were conducted during which the reactor configuration, setup procedure, operation and monitoring procedures, analytical methods, and data generation were examined. Modifications were made to improve the testing process based on the results obtained and observations made during each experiment. Based on the findings, the following conclusions are made.

1. A modified biometer flask approach is the best method for evaluating the amendments. This reactor configuration allows for use of a larger volume of soil, homogenization and better sampling of the soil, and better simulation of in situ venting, and it minimizes the number of atmospheric exchanges and reduces volatile losses.

2. Three experimental conditions are required to effectively evaluate the amendments and compare their performance against indigenous activity. Examining the unamended contaminated soil, the contaminated soil with a sterilized amendment addition, and the contaminated soil with the live amendment added according to the vendor's specifications will provide the necessary data for this evaluation.

3. The hydrocarbon analysis in the soils before and after treatment is the most critical parameter against which to evaluate the performance of the amendments. The TPH measurements and the concentrations of the individual constituents are important and must be included. The soil hydrocarbon data from triplicate reactors should be averaged for comparison purposes.

4. The mass of hydrocarbon removed from the reactors during atmosphere flushing is a small fraction of the mass in the soil but must be accounted for. It is more practical to composite the gas from the three replicate reactors for GC analysis. This will save significant time and, because the mass is relatively small and because the data are to be averaged, compositing will not adversely affect the results.

5. The respiratory measurements are a good surrogate for monitoring microbial activity and can be used to estimate biodegradation rates. However, the results should be used as an indicator only and the final evaluation should be based on the hydrocarbon analysis.

6. Microbial enumeration on the inoculum is important to determine if the amendment contains microorganisms that can degrade hydrocarbons and to determine the inoculation rate. The enumerations on the soils proved inaccurate and were of limited value and should be dropped.

7. The gravimetric organic matter analysis, soil pH analysis, cation exchange capacity analysis, particle size distribution and textural analyses, and alkalinity analysis provide valuable data that will allow for extrapolation of the results between soils with similar characteristics and thus these analyses are important.

8. The analysis of nutrients, including nitrogen, potassium, and phosphorus are important to both evaluate the amendment and to determine if the indigenous activity may be nutrient-limited. It is critical that this analysis be included in the final protocol.

9. Finally, soil moisture data are required to both evaluate microbial activity and convert all data into a dry-weight basis for accurate comparisons. It is critical that this analysis be included in the final protocol.

9.0 PROTOCOL

A detailed description of the reactor setup, experimental methods, and analytical procedures is presented in the protocol contained in Appendix B. The protocol is presented as a standalone document and can be used separate from this report.

10.0 REFERENCES

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APPENDIX A

SOIL CHARACTERISTIC AND NUTRIENT ANALYSIS PROTOCOLS

APPENDIX B

PROTOCOL

FOR

COMMERCIAL MICROBIOLOGICAL AMENDMENT

TESTING AND EVALUATION

PROTOCOL

for

COMMERCIAL MICROBIOLOGICAL AMENDMENT

TESTING AND EVALUATION

to

Air Force Center for Environmental Excellence
8005 9th Street
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TABLE OF CONTENTS

LIST OF TABLES

1.0 INTRODUCTION

The Air Force has identified numerous sites contaminated with petroleum hydrocarbons and is currently involved in an extensive effort to clean up many of these sites. Physical technologies such as soil vacuum extraction and biological technologies such as bioventing have been used successfully to accomplish site closure. Although these technologies are applicable at many sites, there may be sites that can be more readily treated using certain amendments.

A typical amendment product consists of one or any combination of components including a bacterial preparation, a nutrient stock, buffers for pH control, and/or surfactants. Most often, the constituents of the amendments are purchased in a dried state and must be hydrated prior to application. Amendments are added through surface application, by mixing them with excavated soils, or by direct injection into subsurface soils. Application rates are both amendment and site specific and usually are specified by the vendor.

This protocol was developed for the Air Force to use as a tool for evaluating commercially available amendments by determining any enhancement in remediation of petroleum hydrocarbon contamination under controlled conditions. The protocol consists of a series of tests designed to determine any enhancements attributable to the addition of a microbial component or chemical components. The tests involve examining the soil characteristics, the microbial amendment, and the fate of the contaminant.

Certain soil characteristics including moisture, pH, organic matter content, alkalinity, and particle size distribution are important parameters that affect microbial activity and should be measured on all soils that are candidates for amendment treatment.

Microbial activity is strongly influenced by the availability of nutrients, and in soils the supply of necessary nutrients can be low thus limiting contaminant degradation. The more critical nutrients typically are in short supply are nitrogen, phosphorous, and potassium. To properly evaluate microbial amendments, it is necessary to measure the concentrations of these nutrients in the soil both with and without amendment addition. It is also important to measure nutrient concentrations at the end of incubation to determine if the supply of available nutrients became limited during the test.

A number of methods are available for monitoring microbial activity, including respirometry and enumeration. Respirometry is an indirect method that measures microbial activity based on oxygen utilization and carbon dioxide production. Because hydrocarbon degradation is an aerobic process, the oxygen utilization rate can be used to estimate the hydrocarbon degradation rate.

other electron acceptors available?

Enumerating microorganisms that can degrade target contaminants is a valuable tool for determining the contribution of microbes with the desired capability that a specific microbial amendment provides to soils. It is important to conduct the enumeration analysis to determine the number of hydrocarbon-degrading bacteria in the inoculum to quantify the number of these organisms that are added to the soil during inoculation.

The most important parameter used to compare the effectiveness of an amendment against indigenous microbial activity is the reduction in contaminant. It is necessary to measure the hydrocarbon content of the soil before and after treatment and in the gas exchanged during reactor operation. If an amendment is to be deemed effective, the hydrocarbon reduction in the amended soil must significantly exceed the reduction in the unamended soil.

The following sections contain detailed descriptions of the preparation, experimental setup, monitoring activities, and analytical methods to be conducted to perform an evaluation under controlled conditions. These methods have been selected based on the results of extensive testing and analyses during protocol development experiments. The suite of analyses will provide the information necessary to determine if the use of the amendment would provide any significant enhanced remediation capacity over that of indigenous microorganisms.

2.0 OBJECTIVE

The objective of this protocol is to provide a standardized testing and evaluation procedure that can be used by the Air Force to screen commercially available microbial amendment products. The objective of testing and evaluating commercial microbial amendments is to demonstrate, under carefully controlled conditions, whether these amendments provide a significant improvement over indigenous microorganisms in the biodegradation of petroleum hydrocarbons.

3.0 EXPERIMENTAL METHODS

3.1 Reactor Design

The tests are conducted using a reactor flask design. Each reactor consists of a 500-mL wide-mouth Erlenmeyer flask fitted with a Neoprene™ rubber stopper. Two 18-gauge stainless steel Luer lock needles are inserted through the rubber stopper to facilitate sampling and exchanging reactor atmospheres.

One of the needles is 6 inches long and is placed so that the tip is below the soil surface and just above the bottom of the flask. This needle serves as the influent line for introducing air during atmospheric exchanges. The second needle is approximately 2 inches long and is placed so that the tip is just below the bottom of the rubber stopper. This needle is used to extract gas during sampling and atmospheric exchanging.

The head of each needle is equipped with a two-way Luer lock valve to provide a mechanism for both removing gas and sealing the reactors. The valves are designed for syringe attachment. This design allows gas exchange to closely simulate conditions seen in bioventing and/or biopile configurations.

3.2 Experimental Conditions

Three experimental conditions are established to evaluate the performance of the amendment product. Triplicate reactors are set up for each condition using a common batch of contaminated soil. The conditions examined include contaminated soil without any amendment, contaminated soil with a filter-sterilized amendment preparation, and contaminated soil with an amendment added according to the vendor's specifications.

The contaminated soil without amendment provides the baseline degradation data against which the amendment is compared. Because nothing is added to the soil under this condition, any degradation is the result of indigenous microorganisms. The results from this condition are representative of the degradation that might be obtained during treatment without amendment addition.

The contaminated soil with the sterilized amendment addition provides data on any contribution from the nonbiological components of the amendment product to the biodegradative capacity of the indigenous microorganisms. Added nutrients could provide these microorganisms with a needed supply to support a higher rate of activity than is seen under the nonamended condition. Surfactants often are a component of amendment products, and their addition could increase the bioavailability of

certain contaminant compounds and increase their biodegradation rate. The data from this condition are compared against the data from the nonamended condition to determine if there are enhancements.

The final condition is set up with contaminated soil and the amendment product added according to the vendor's specifications. The preparation procedure for the product, the application rate(s), and any special application directions must be obtained from the vendor. It is critical that vendors provide this information to ensure that the products are properly evaluated. Vendors that do not provide clear and concise directions stand the chance of having their products fail the evaluation, and the argument that a product was not evaluated properly cannot be accepted.

3.3 Soil Processing and Amendment Addition

The procedures for soil processing and amendment addition are described in the following paragraphs.

Prior to preparing the soils, weigh and label three porcelain crucibles as described in Section 4.7 below. Label one large Tedlar™ soil sampling bag and three smaller bags. Prepare all materials, supplies, and media for all of the analytical methods described in Section 4.0, accordingly. Once these preparations are complete, prepare the soil as follows.

Prepare the soil in two steps. First, transfer an amount of soil sufficient to accommodate the number of reactors under all three conditions into the large Tedlar™ soil sampling bag. Seal the bag and hand-knead until the contents appear homogeneous. This step is extremely important because the soil will be segregated into the stocks for the three conditions. Once the soil appears homogeneous, remove an aliquot, analyze it by gas chromatography (GC) (see Section 4.1), and record the TPH value. During the analysis, continue to knead the bag and pull a second sample for TPH analysis. Repeat the kneading process for a third time, and again measure the TPH in the soil. This process is repeated until the coefficient of variance (CV) of three consecutive TPH values is 10% or less. Once this level of homogeneity is achieved, the soil is ready for the next step.

Following the kneading process described above, weigh triplicate 10-g samples into each of the three preweighed and labeled porcelain crucibles described above and conduct a baseline moisture content analysis of the homogenized soil (see Section 4.7). Once the moisture content is determined, the soil can be segregated into the three smaller Tedlar™ bags to be readied for the three experimental conditions. Divide the homogenized soil into three separate stocks, making sure to transfer enough soil to meet the requirements for each condition.

Prepare the amendment according to the vendor's guidelines. The amendment dosage and moisture content are determined based on the vendor's specifications for the particular microbiological amendment product. If the vendor does not provide moisture content recommendations, prepare the amendment to achieve a final moisture content of 20%. Once the amendment is prepared, divide the stock into two equal parts.

Sterilize one part of the amendment by filtering it through a 0.22-μm membrane filter into a sterilized receiver. This stock will be used to prepare the contaminated soil with sterilized amendment condition. In addition, filter-sterilize an equal volume of distilled (or equivalent grade) water to use for moisture adjustment in the nonamended condition.

Add the required amount of the appropriately prepared amendment to the volume of soil in the bags for each respective condition. Add the volume of sterilized water necessary to achieve the targeted moisture content to the soil in the bag not receiving amendment. Seal the bags, taking care to minimize headspace.

Thoroughly hand-knead the soils in the bags until the contents are well mixed (up to 1 hour). Transfer an appropriate volume of each soil into clean, prelabeled containers to conduct the initial soil analyses listed in Section 4.0.

3.4 Reactor Setup

Label a reactor flask with the amendment identification (ID), the experimental condition, the replicate number, the date of setup, and the initials of the preparer. Tare the flask on a two-place balance. Transfer enough of the appropriate homogenized soil into the reactor to fill the flask to the 200-mL mark and record the soil weight. Continue this procedure and fill the remaining reactors. Place the stopper with the needles in place into the mouth of each flask. Make sure that the longer needle is situated just above the bottom of the flask. Insert the stopper so that a tight seal is established and check to make sure that the valves on each flask are closed. Place the reactors in an upright position in a temperature-controlled incubator maintained at 25°C unless an alternative temperature is specified by the vendor and/or the Air Force.

3.5 Reactor Operation

3.5.1 Maintaining Temperature. The temperature on the incubator is checked on a regular basis to ensure that the reactors are kept at the desired temperature. In the event that the temperature varies by more that a few degrees, the incubator is readjusted to the appropriate setting.

3.5.2 Atmosphere Exchanges. To maintain an aerobic environment in each reactor, it is necessary to regularly exchange the reactor atmospheres. The exchanges are made approximately once per week; however, the required exchange rate must be determined by measuring the oxygen concentrations in the reactor headspace (see Section 4.3). The atmospheres are exchanged when the oxygen concentrations drop below 10%.

The atmospheres are exchanged using a direct-flush method. Fill a 1-L syringe with 300 mL of clean air and connect the syringe to the valve on the long needle. Connect a 500-mL Tedlar™ gas sampling bag to the valve on the shorter needle. Open the valve on the Tedlar™ bag, then open the valve on the shorter needle. Next, open the valve on the longer needle and slowly push the 300 mL of air from the syringe through the rector. As the air is forced from the syringe, the Tedlar™ bag will inflate. Allow the reactor to come to equilibrium at ambient pressure. Close all of the valves on the reactor and the valve on the Tedlar™ bag. Remove the syringe and the bag. The gas in the bag is ready for analysis.

3.6 Process Monitoring

Oxygen and carbon dioxide concentrations are measured in the gas collected from each reactor during atmosphere exchanges (see Section 4.3). The data are used to monitor the oxygen concentrations to ensure that oxygen levels do not become rate-limiting and to estimate biodegradation rates. The final evaluation of biodegradation performance of an amendment under each of the experimental conditions is assessed using TPH data from analyses of initial and final soil samples (see Section 4.1).

The parameters listed in Table 1 are measured in the initial soil samples collected from the homogenized batches of soil at the time of reactor setup. The data are used as the baseline against which to evaluate the biodegradation in each of the reactors. The final soil samples are collected from each reactor after 30 days of incubation. These samples are analyzed for the parameters listed in Table 1. The baseline data are subtracted out, and the resulting data for each experimental condition are compared.

3.7 Reactor Harvesting

After the 30-day incubation period, the reactors are harvested and the soil is removed for analysis. Each reactor is harvested individually to maintain the integrity of the sample and to minimize the potential for cross-contamination between reactors.

Table 1. Sampling Schedule for Testing and Evaluating Commercial Microbiological Amendments.

Analysis	Initial	Intermediate	Final
Soil pH	S		S
Soil Moisture	S		S
Alkalinity	S		S
Particle Size Distribution	S		
Textural Analysis	S		
Cation Exchange Capacity	S		
Organic Matter Content	S		S
Nutrients (N,P,&K)	S		S
Microbial Enumerations	S,I		
Oxygen and Carbon Dioxide	G	G	G
Hydrocarbon in Gas	G	G	G
Hydrocarbon in Soil	S		S

S = Soil Samples
I = Inoculum
G = Gas Samples

Before removing the soil from a reactor, lay out a piece of clean nonabsorbent or wax paper to contain the soil sample if it is spilled. The paper should be discarded and replaced with a clean piece after harvesting each reactor. Prepare all materials, supplies, and media needed for all of the analyses to be conducted on the soils collected during harvesting. Label all transfer bottles, tubes, and vials appropriately to facilitate the harvesting process.

Use a clean stainless steel spatula to remove any soil from the reactors. Clean the spatulae with distilled water, rinse with ethanol, then flame it over a Bunsen burner between each reactor harvest to reduce carryover of contaminants, soil constituents, and microorganisms between samples. Allow the spatula to cool to room temperature before inserting it into the next reactor.

Transfer soil samples into clean acid-washed bottles or vials and seal with Teflon™-lined caps. Analyze samples as soon as possible. Harvest each reactor in a consistent manner to ensure proper sampling and sample handling techniques are followed.

To harvest the reactors, remove the flask stopper and retrieve the soil sample with the sterilized stainless-steel spatula. After each soil removal reset the stopper into the flask.

First, remove approximately 3 mL of soil and transfer it into a clean 3-mL Teflon™-lined screw-cap vial. Fill the vial to the top in order to limit the amount of headspace and volatile loss of hydrocarbon. Seal the vial as quickly as possible. Submit this sample for soil TPH analysis.

Next, remove approximately 10 g of soil and transfer it into a preweighed crucible with lid. Place the lid on top of the crucible and immediately record the weight of the sample and crucible with lid. Use these samples for moisture content analyses and subsequently for organic matter analyses.

Remove approximately 5 g of soil from the reactor and place the soil into a 20-mL glass screw-cap scintillation vial. Add 5 mL of Milli-Q grade water and screw the cap back onto the vial. This vial will be used for pH analysis. Finally, transfer the remaining soil into an I-Chem® bottle for nutrient and soil properties analyses.

4.0 ANALYTICAL METHODS

4.1 Petroleum Hydrocarbon Concentrations in Soil Samples

Soil samples are analyzed on a Hewlett-Packard (HP) 5890 GC using a heated purge-and-trap method. The GC is equipped with a 30-m DB-1 wide-bore capillary column connected to a flame ionization detector (FID). The initial oven temperature is held at 20°C with cryogen for 4 minutes, then ramped at 10°C/min to 240°C and held for 4 minutes or until a stable baseline is achieved. Peak elution along with resulting area counts are recorded and stored as computerized files using the Chromperfect® for Windows data acquisition software package. The concentrations of the 20 compounds listed in the calibration mixture are calculated by applying response factors determined from responses from injections of known concentration.

Weigh out approximately 1 g of soil into a test tube and add 5 mL of Milli-Q water. Attach the test tube to the purge-and-trap concentrator fitted with the sterilized soil sparging needle. Purge the sample for 8 minutes at 85°C and collect the purged hydrocarbons on the sorbent trap. Desorb the trapped organics to the GC for compound resolution and quantification.

4.2 Petroleum Hydrocarbon Concentrations in Gas Samples

Gas samples collected during atmospheric exchanges are analyzed for petroleum hydrocarbons. After the three gas samples of each condition are processed for oxygen and carbon dioxide

concentrations, 50 mL of each of the gas samples are pulled by a 200-mL gastight syringe and are injected into one Tedlar™ sampling bag to create a composite sample of each of the three conditions. By creating this composite sample bag at the analytical stage rather than averaging the three values at the data reduction stage, the analysis time of the samples is cut by two-thirds. An HP 5890 GC equipped with a 60-m DB-1 wide-bore capillary column (J & W Scientific) connected to a FID is used to analyze the composite gas samples for petroleum hydrocarbons. An auto sampler attached to the six-port valve injection port equipped with a heated sample loop is used to introduce 2 mL of the sample into the GC. The oven temperature is held at 20°C for 4 minutes, then ramped at 10°C/min up to 180°C and held for 12 minutes. The data are collected by Chromperfect® and the concentrations of the specific hydrocarbons are calculated using Chromperfect® by multiplying the resulting area count for each compound by the response factor. The response factors are calculated by dividing known concentrations of each of the 20 compounds by their respective area counts. The area counts are determined by injecting and analyzing a calibration standard at that concentration.

4.3 Oxygen and Carbon Dioxide Concentrations in Gas Samples

Oxygen and carbon dioxide are measured in the gas collected from each reactor flask during the atmosphere exchanges. When gas samples are collected, analyses for oxygen and carbon dioxide concentrations are performed prior to petroleum hydrocarbon gas analysis. The oxygen concentration data are used to ensure that the oxygen levels do not become rate-limiting and to determine the biodegradation rates over the incubation period. An SRI GC equipped with a CTR-I concentric column (Altech) connected to a thermal conductivity detector (TCD) is used to analyze the gas samples for oxygen and carbon dioxide concentrations. An isothermal method at ambient temperature is used, with helium serving as the carrier gas. A 10-mL sample volume is injected through a multiport valve injector assembly which contains a 2-mL looped system. The concentrations of oxygen and carbon dioxide are calculated using response factors generated from a multipoint calibration from injections of standards of known concentrations.

4.4 Microbial Enumerations in Inoculum

Initial microbial enumerations are conducted on the microbial amendment inoculum to determine the number of cells/g dry soil added. Enumerations of the amendment are conducted in triplicate using a serial dilution and spread-plate method. The procedure for this analysis is as follows:

Step 1. Prepare 0.2M solutions of K_2HPO_4 and KH_2PO_4 in distilled water. Mix the solutions in the ratio 77 parts K_2HPO_4 to 28 parts KH_2PO_4. Adjust the pH to 7.2 using KOH or HCl. Dispense 9-mL aliquots of the potassium phosphate buffer into 30-mL borosilicate glass test tubes for dilution blanks. Cap and autoclave for sterilization. After sterilization, store the tubes in a refrigerator at 10°C until they are ready to be used. Allow the tubes to equilibrate to room temperature before using.

Step 2. Prepare a basal inorganic medium by adding 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.05 g $CaSO_4 \cdot 2H_2O$, 0.5 $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 1.0 g $(NH_4)_4SO_4$ into 1.0 L of distilled water and adjust the pH to 7.2 using KOH or HCl. Add 20 g noble agar (purified agar) to the medium and stir with heat until the agar has dissolved. Autoclave for sterilization. After sterilization, dispense 20-mL aliquots into petri dishes. After the medium has solidified in the petri dishes, label the bottom of the petri dishes with the dilution and replicate information.

Step 3. Aseptically transfer 1 mL of prepared amendment (prepared according to the company's directions) into triplicate tubes containing 9 mL of dilution blank. Vortex for 10 seconds. Aseptically transfer 1 mL of this suspension to a dilution blank containing 9 mL of buffer and vortex for 5 seconds. Continue with this dilution process until the desired number of dilutions have been made.

Step 4. Aseptically transfer 0.1 mL of each dilution to the appropriate plate. Spread using a glass spreader that has been sterilized with ethanol and then flamed. Allow the flame on the glass spreader to go out by itself to ensure all ethanol has been burnt off. Also take care to use the glass spreader only after it has cooled to ensure that the microbes are not killed by the heat. Invert the plates and place inside a gas pack. Place an absorbent pad with 1 mL of JP-4 jet fuel into the gas pack and seal the gas pack. Incubate at 25°C until colonies appear and then count the colonies.

4.5 Soil Moisture

Initial and final soil moisture content analyses are conducted to determine whether the soils contain suitable water content to support microbial growth and nutrient transfers, to monitor the net water loss by the end of the incubation period, and to determine a constant that is used in soil dry-weight calculations. A gravimetric method (Gardner, 1965) is used to make the moisture determination. Soil moisture analyses are conducted as follows:

Step 1. Turn on the drying oven and allow the temperature to equilibrate at 105°C.

Step 2. Preweigh crucibles with crucible lid and label each by etching both with an ID number.

Step 3. Weigh out approximately 5 g of soil in triplicate and place in the preweighed, labeled crucibles. Cover the samples with the respective crucible lid.

Step 4. Place covered crucibles containing the soil samples into the drying oven and allow them to dry for 24 hours.

Step 5. Remove the crucibles from the drying oven and place them into a desiccator at room temperature. Allow the crucibles to cool to constant weight at room temperature.

Step 6. Remove the crucibles from the desiccator and record the cumulative weight of the crucible plus the sample after drying.

Step 7. Calculate the percent soil moisture content with the following formula:

$$\% \text{ moisture content} = \frac{(\text{weight of soil})_{wet} - (\text{weight of soil})_{dry}}{(\text{weight of soil}_{dry} - \text{tare})} \times 100 \qquad (1)$$

4.6 Organic Matter Content in Soil Samples

Organic matter is measured in initial and final soil samples to characterize the soil samples and to monitor carbon loss due to biodegradation. The analysis is conducted on the samples used to determine the soil moisture content as follows.

Step 1. Turn on the muffle furnace and equilibrate temperature at 550°C.

Step 2. After the final cumulative weights are determined in Step 6 of the soil moisture content procedure, place the covered crucibles with sample into the muffle furnace and close the muffle furnace door. Allow the samples to combust at 550°C for 1 hour.

Step 3. After 1 hour remove the crucibles from the muffle furnace and immediately place the dishes into a desiccator. Allow the crucibles to cool to constant weight at room temperature.

Step 4. Remove each crucible from the desiccator and record the cumulative weight of the dish plus the sample after muffling.

Step 5. Calculate the soil organic matter content using the following formula:

$$\text{organic matter content (\%)} = \frac{(\text{weight of soil})_{dry} - (\text{weight of soil})_{combust}}{(\text{weight of soil})_{dry}} \times 100 \qquad (2)$$

4.7 Nutrient Concentrations in Soil Samples

Initial and final soil samples are analyzed for calcium, magnesium, potassium, phosphorus, and nitrogen. The following is the detailed procedure used to measure each analyte:

4.7.1 Calcium, Magnesium, and Potassium. Follow the procedure below to measure the calcium, magnesium and potassium concentrations in soil samples. The volumes may be reduced depending on soil availability.

Prepare the following reagents:

1. Extracting Solution: Dispense approximately 100 L of water into a 30-gal reagent tank calibrated to 115 L. Add 6,560 mL of concentrated acetic acid. Then add 7,900 mL of concentrated ammonium hydroxide. Allow the solution to cool and adjust the pH to 7.0 with acetic acid or ammonium hydroxide. Bring the volume to 115 L and mix. The pH of this solution should be monitored.

2. Stock Standards: Ca = 10,000 ppm; Mg = 1,000 ppm; K = 1,000 ppm.

3. Working Standards:*

   a. Ca = 2,000 ppm and Mg = 300 ppm: Add 200 mL of Ca Stock Standard and 300 mL of Mg Stock Standard to a 1,000-mL volumetric flash, and dilute to volume with Extracting Solution.

   b. Ca = 1,000 ppm and Mg = 150 ppm: Dilute 250 mL of Standard A to 1,000 mL with Extracting Solution.

   c. Ca = 500 ppm and Mg = 75 ppm: Dilute 250 mL of Standard A to 1,000 mL with Extracting Solution.

   d. K = 25 ppm: Add 25 mL of the K Stock Standard to a 1-L volumetric flash and dilute to volume with deionized water.

   e. K = 50 ppm: Dilute 50 mL of the K Stock Standard to 1,000 mL with deionized water.

   f. K = 100 ppm: Dilute 100 mL of the K Stock Standard to 1,000 mL with Extracting Solution.

*Note that the potassium standards have been made in water rather than extracting solution.

Use the following procedure to prepare the sample and analyze for the listed nutrients.

Step 1. Measure 5 g of soil into a 20-dram vial using a standard NCR-13 scoop.

Step 2. Dispense 25 mL of Extracting Solution. Seal and shake for 15 minutes at 180 rpm.

Step 3. Filter through Whatman #2 paper and filter if the extract is cloudy.

Step 4. Determine the Ca, Mg, and K levels by atomic absorption.* A blank must be carried through with each set. (ppm in the soil = 5 × ppm in the extract.)

4.7.2 Phosphorous. Use the following procedure to measure phosphorus in soils. First prepare the reagents listed below.

1. Weak Bray Solution: Weigh 127.7 g of ammonium fluoride into a 30-gal Nalgene tank marked for 115 L. Add approximately 30 L of deionized water. Add 240 mL of concentrated HCl. Dilute to 115 L with deionized water and mix well with an electric mixer.

2. Strong Bray Solution: Weigh 127.7 g of ammonium fluoride into a 30-gal Nalgene tank marked for 115 L. Add about 30 L of deionized water. Add 958 mL of concentrated HCl and dilute to 115 L with deionized water, mixing with an electric mixer.

3. P_2 Solution (Molybdate): Weigh 100 g of ammonium molybdate crystals into a 1,000-mL graduated cylinder. Add deionized water to 850 mL. Mix until the solution is homogeneous. Add this solution to the P_2 reagent bottle, and add 1,700 mL of concentrated HCl with mixing.

4. P_3 Solution (Color Developing Reagent): Place 50 g of sodium sulfite and 25 g of 1-amino-2-naphthol-4-sulfonic acid in a large mortar and grind thoroughly until the color is evenly distributed. Add 1,463 mL of sodium bisulfite. Grind and mix again until the color is uniform. Dry overnight at 105°C. Prepare the solution by dissolving 27.0 g of the dry mixture in distilled water, and dilute to 1,000 mL. (A warm water bath may facilitate dissolution.) Make this solution once per week as it has limited stability.

5. Stock Phosphorus Solution (100 ppm): Dissolve 0.4394 g of dried KH_2PO_4 in approximately 250 mL of deionized water in a 1,000-mL volumetric flash.** Dilute to volume and mix well.

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*The source recommends use of flame emission spectroscopy for determination of K.

**_Methods of Soil Analysis: Part 2 – Chemical and Microbiological Properties_, 2nd ed. (ASA, 1982), recommends the addition of five drops of toluene to the phosphorus stock standard to diminish the possibility of microbial activity.

6. Working Phosphorus Standards (1 ppm, 5 ppm, 10 ppm): Accurately pipette 10 mL of the stock standard into a 1,000-mL volumetric flash. Dilute to volume, and mix well. Follow this same procedure using 50 mL and 100 mL to make the 5-ppm and 10-ppm working standards. Do not add the aliquot of stock standard after bringing to volume. The final volume must be 1,000 mL.

Use the following procedure.

Step 1. Weak Bray Extraction:

a. Measure 1 g of soil into the phosphorus extraction cup.

b. Add 7 mL of Weak Bray Solution to the cup and shake for 1 minute.

c. Filter through a medium retention filter paper into small phosphorus vials. Develop the color and read on the Gilford as outlined below.

Step 2. Strong Bray Extraction:

a. Measure 1 g of soil into a phosphorus extraction cup.

b. Add 7 mL of Strong Bray Solution to the cup and shake for 1 minute.

c. Filter the extract through a medium retention filter into small phosphorus vials.

Step 3. Color Development:

a. Refilter any extract that is cloudy. All extracts must be clear.

b. Set up the standard curve by adding 4.5 mL of deionized water, 4.5 mL of the 1- ppm standard, 4.5 mL of the 5-ppm standard, and 4.5 mL of the 10-ppm standard into small phosphorus vials, and proceed with the color development.

c. Add 0.3 mL of P_2 solution to each standard, blank, and soil extract using the Oxford Adjustable Dispenser.

d. Add 0.3 mL of Pb_3 to each standard, blank, and soil extract using another Oxford Adjustable Dispenser.

e. Let stand for 30 minutes before reading to ensure complete color development.

f. Set up a standard curve on the Gilford Spectrophotometer at 660 nm in the direct concentration mode at the following settings:

Blank = 0000
1 ppm = 0070
5 ppm = 0350
10 ppm = 0700

Notes:

(1) The dispensers used for the weak and strong brays must be checked daily to ensure that the correct volume (7 mL) is being dispensed. They should also be checked while in use.

(2) The volumes of P_2 and Pb_3 solutions being dispensed should also be checked daily and throughout the procedure.

(3) The P_2 (Molybdate) Solution is made from concentrated HCl. Use the eye protection provided and avoid contact with the skin. Keep the area well ventilated.

4.7.3 Total Nitrogen. Use the following procedure to measure total nitrogen in the soil samples. First prepare the following reagents.

1. Potassium Sulfate-Catalyst Mixture: Prepare a mixture of 200 g of potassium sulfate, 20 g of cupric sulfate pentahydrate, and 2 g of Se. Powder the reagents separately, and grind the mixture in a mortar to powder the cake that forms during mixing.

2. Concentrated and Standardized 0.1N Sulfuric Acid.

3. Sodium Hydroxide Solution ($\simeq$ 10N): Place 3.2 kg of reagent-grade NaOH in a heavy-walled 10-L Pyrex™ bottle marked up to 8 L. Add 4 L of carbon dioxide-free water, and swirl the bottle until the alkali is dissolved. Cool the solution with a rubber stopper in the neck of the container, and bring it to 8 L with carbon dioxide-free water. Swirl vigorously, and store the bottle with some arrangement that allows the alkali to be stored and dispensed with protection from CO_2.

4. Mixed Indicator Solution: Dissolve 0.099 g of Bromcresol Green and 0.066 g of Methyl Red in 100 mL of ethanol. Now add 0.1N NaOH cautiously until the solution assumes a reddish-purple tint (pH $\simeq$ 5). Make the solution to 4 L by adding water. Mix thoroughly.

5. Boric Acid-Indicator Solution: Place 80 g of boric acid in a 5-L flask marked to indicate a volume of 4 L. Add 3,800 mL of water and heat and swirl the flask until the boric acid is dissolved. Cool the solution and add 80 mL of the mixed indicator solution.

Use the following procedure to prepare and analyze the soils.

Step 1. Place a sample expected to contain about 1 mg of nitrogen in a micro-kjeldahl digestion flask, add 1.1 g of the potassium sulfate-catalyst mixture and 3 mL of concentrated sulfuric acid, and heat the flask cautiously on the digestion stand.

Step 2. When frothing has ceased, increase the heat until the digest clears, and then adjust the heat so that the reflux line for the sulfuric acid appears about one-third of the way up the neck of the digestion flask. Continue the digestion for 5 hours.*

Step 3. Allow the flash to cool, and slowly add 20 mL of water. Swirl to suspend solids, and transfer the contents to the distillation chamber of the Hoskins apparatus via the funnel. Rinse the kjeldahl flash 3 times with a total of about 9 mL of water and add the rinsings to the digestion flash. Bring the solution level up to a mark made previously to indicate a volume of 50 mL, and close the stopcock connecting the funnel and distillation chamber.

Step 4. Add 5 mL of boric acid-indicator solution to a 50-mL Erlenmeyer flash previously marked to indicate a volume of 35 mL, and place the flash under the condenser so that the end of the condenser is about 4 cm above the solution.

4.8 Soil pH

The soil pH is determined before and after incubation. The initial soil pH values are determined for each experimental condition before incubation and soil pH is determined after the incubation period for each reactor. The pH of the soils is determined as follows:

Step 1. Calibrate the pH-meter using a dual-point calibration with pH 4.00 and pH 7.00 standards. Check the calibration slope to make sure the probe is in good working order.

Step 2. Add approximately 5 g of soil to 5 mL of Milli-Q water in a prelabeled 20-mL screw-cap glass scintillation vial.

Step 3. Screw the cap onto the vial and allow the slurry to mix on a bench-scale shake table at 200 rpm for 4 days. This will provide ample time for equilibration of the soil/water matrix.

Step 4. After the 4-day equilibration period, remove the cap from the vial and insert a small magnetic stir bar. Place the vial with stir bar onto a stir plate and allow to mix for 15 seconds.

Step 5. Place the pH electrode into the soil/water suspension and record the displayed pH value.

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*The ammonia-nitrogen produced by digestion for 2 hours is rarely less than 98% of that formed in the recommended 5 hours. According to the source, even a 1-hour digestion period is adequate for routine soil analyses not requiring high degrees of accuracy.

4.9 Physical Properties of Soil Samples

Physical soil characteristics including such as particle size distribution and CEC are important parameters that affect microbial activity and will be measured on all initial soils samples. In addition the data is used to extrapolate the results from an amendment test to soils having similar characteristics from other sites.

4.9.1 Particle Size Distribution and Textural Analyses. Particle size distribution analysis is conducted for the initial soils. Soil particle size and percent sand, silt, and clay are determined using timed hydrometer readings in a modified Stoke's law equation. Soil texture is determined by comparing particle size with the U.S. Department of Agriculture (USDA) soil texture triangle. A detailed description of the method for particle size distribution analysis follows:

Prepare or obtain the following item and reagents:

1. Sodium Hexametaphosphate Solution: Dissolve 900 g of sodium hexametaphosphate in 18 L of distilled water.

2. Isopropyl Alcohol.

3. Hydrometer: ASTM No. 152H with Bouyoucos scale in g/L, T=67°F.

Use the following procedure to determine particle size:

1. Tag a plastic cup with a sample number. Then tare the plastic cup and weigh out 50 g of sample (25 or 10 g may be used if there is not enough sample; 100 g should be used for sandy soils). Record the weights.

2. Transfer the number tag to a blender cup. Add the sample to 20 mL of sodium hexametaphosphate solution. Dilute to 100 mL with distilled water adjusted to 67°F. Blend for 5 minutes.

3. Start the stopwatch and adjust the sink temperature to 67°F.

4. Transfer the number tag to a 1,000-mL cylinder, and wash the sample into the cylinder with distilled water, bringing the total volume to 1,000 mL.

5. Stopper the cylinder and thoroughly mix the contents by inverting it several times while shaking. Add one drop of isopropyl alcohol if the mixture is considerably foamy.

6. Set the cylinder on the table and record the starting time. Place the hydrometer and thermometer into the suspension, and read both instruments exactly 40 seconds after the starting time.

7. Let the cylinder stand for 2 hours. Replace the hydrometer into the suspension and again read the hydrometer at water level. Record the reading and the temperature. Rinse the cylinder.

8. Take the results to the computer for analysis of percent sand, percent silt, and percent clay, and use these values to name the soil texture according to the USDA soil texture triangle diagram supplied with this method.

Make the following hydrometer adjustments and calculations:

1. Hydrometer Correction: Because the hydrometers are calibrated at 67°F, the temperature dependency of Stoke's law must be adjusted for variations in the experimental temperatures. According to L.D. Baver, *Soil Physics*, (3rd ed.), "the (terminal) velocity of fall at 30°C is about 12 percent faster than at 25°C." The Iowa State University soil science lab manual suggests that this relationship between temperature and terminal velocity yields the following hydrometer temperature dependency:

> "For each degree above the calibration temperature, add 0.2 g of soil to the reading to get the corrected hydrometer reading. For each degree below the calibration temperature, subtract 0.2 g of soil from the reading."

Therefore, to get the actual hydrometer reading, incorporate the temperature correction as stated above, and subtract the following experimentally determined blank readings:*

Temp.	Subtract
66-68°F	0
61-65°F	1
55-60°F	2

2. Percent Sand:

For 50-g samples:

$$\% \text{ Sand} = 100 - (\text{Corrected 40-second hydrometer reading}) \times 2.0$$

For 25-g samples:

$$\% \text{ Sand} = 100 - (\text{Corrected 40-second hydrometer reading}) \times 4.0$$

For 100-g samples:

$$\% \text{ Sand} = 100 - (\text{Corrected 40-second hydrometer reading})$$

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*This linear temperature correction is probably only valid over a small range of temperatures (i.e., ±3°F from the calibration temperature).

3. Percent Clay:

For 50-g samples:

$$\% \text{ Clay} = (\text{Corrected 2-hour hydrometer reading}) \times 2.0$$

For 25-g samples:

$$\% \text{ Clay} = (\text{Corrected 2-hour hydrometer reading}) \times 4.0$$

For 100-g samples:

$$\% \text{ Clay} = \text{Corrected 2-hour hydrometer reading}$$

4. Percent Silt:

$$\% \text{ Silt} = 100 - (\% \text{ Sand} + \% \text{ Clay})$$

4.9.2 Cation Exchange Capacity. Initial soil samples are analyzed for their cation exchange capacity (CEC) by using an ammonium saturation method. Soil is leached with an excess of neutral 1N ammonium acetate solution to remove the exchangeable cations and to saturate the exchange material with ammonium ions. After removal of excess ammonium present in the soil as the acetate, the exchangeable ammonium is determined by displacement with NaCl and distillation. The following is a detailed description of this method.

To prepare the reagents:

1. Saturating Solution (Ammonium Acetate, 1N): Dilute 114 mL of glacial acetic acid (99.5%) with distilled water to a volume of approximately 1 L. Add 138 mL of concentrated ammonium hydroxide and dilute to approximately 1,980 mL. Adjust to pH 7.0 with ammonium hydroxide or acetic acid. Dilute to 2 L with distilled water.

2. Isopropyl Alcohol (99%).

3. Ammonium Chloride (1N): Adjust to pH 7.0 with ammonium hydroxide.

4. Ammonium Chloride (0.25N): Adjust to pH 7.0 with ammonium hydroxide.

5. Ammonium Oxalate [$(NH_4)_2C_2O_4 \cdot H_2O$, 10%].

6. Dilute Ammonium Hydroxide: Add 1 volume of concentrated ammonium hydroxide to an equal volume of distilled water.

7. Silver Nitrate (0.10N).

8. Sodium Chloride (acidified): Prepare a 10% aqueous solution of NaCl (ammonia-free) by dissolving 100 g of NaCl in 990 mL of distilled water. Add 0.5 mL of HCl (or enough HCl to bring the solution to a pH of approximately 5.3).

9. Sodium Hydroxide (1N).

10. Boric Acid (2%).

11. Standardized Sulfuric Acid (0.1N).

12. Bromcresol Green-Methyl Red Mixed Indicator Solution: Tritate 0.1 g of Bromcresol Green with 2 mL of 0.1N NaOH in an agate mortar, and add 95% ethanol to obtain a volume of 100 mL. Tritate 0.1 g of Methyl Red with a few mL of 95% ethanol in an agate mortar. Add 3 mL of 0.1N NaOH, and dilute the solution to a volume of 100 mL with 95% ethanol. Mix 75.0 mL of the Bromcresol Green solution with 25.0 mL of the Methyl Red solution. Dilute to 200 mL with 95% ethanol.

Use the following procedure to determine the CEC of the soil:

1. Place 10 g* of air-dried soil in a 500-mL Erlenmeyer flask and add 250 mL of neutral, 1N ammonium acetate solution.

2. Shake the flask thoroughly and allow it to stand overnight.

3. Filter the soil by light suction using a 55-mL Buchner funnel. Do not allow the soil to dry or crack.

4. Leach the soil with the neutral ammonium acetate until the solution is negative on the calcium test.

   Calcium Test:

   a. Place 10 mL of the leachate in a test tube and add a few drops of 1N ammonium chloride.

   b. Add also a few drops of 10% ammonium oxalate and dilute ammonium hydroxide.

   c. Heat to near boiling.

   d. Calcium is indicated by turbidity or white precipitate.

5. When the calcium test is negative, save the leachate for the exchangeable base determination, or discard.

6. Leach the soil four times with neutral 1N ammonium chloride and once with 0.25 ammonium chloride.

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*Use 25.0 g of sample if the CEC is expected to be very low (3 to 5 m_{eq} per 100 g).

7. Wash out the electrolyte with 150 to 200 mL of 99% isopropyl alcohol until the test for chloride in the leachate (using 0.1N silver nitrate) is negligible.

8. Leach the ammonium-saturated soil with 10% NaCl (acidified) by small portions until 225 mL have passed through the sample. Allow each portion to pass through before adding the next.

9. Transfer the leachate quantitatively to an 800-mL kjeldahl flask. Add 25 mL of 1N NaOH and distill 60 mL of the solution into 50 mL of 2% boric acid.

10. Add 10 drops of the Bromcresol Green-Methyl Red mixed indicator solution and titrate with standardized 0.1N sulfuric acid.*

11. Run blanks on the reagents.

12. Correct the titration figure for the blanks, and calculate the m_{eq} of ammonium in 100 g of soil.

Use the following formula to calculate the CEC:

Cation Exchange Capacity (CEC) = m_{eq} of NH_4 per 100 g of sample (m_{eq}/100 g).

5.0 DATA ANALYSIS AND AMENDMENT EVALUATION

The data produced from the analyses conducted under this protocol are used to evaluate the performance of the amendment product against the degradation performance of the indigenous microorganisms without the addition of the amendment. Analyzing the data will allow the determination of any potential enhancements that the amendment provides for a given soil and soil type. The data are analyzed as follows.

5.1 Soil Characteristic Data

The soil characteristic data from the pH, particle size distribution, CEC, organic matter, and alkalinity analyses are used to extrapolate the results from an amendment test to soils having similar characteristics from other sites, to screen potential candidates for further testing for application at that site.

5.2 Microbial Enumeration Data

*The color change for this titration is from bluish-green to bluish-purple.

The data from the enumeration of the microorganisms in the inoculum are used to verify the presence of viable microorganisms that are capable of degrading hydrocarbon. The data also are used to determine the rate at which live microbes are added to the soil. These data can be useful for evaluating rates between amendments on a organism-dosing basis. Amendments that show little or no growth during the enumeration analysis and that do not provide any enhanced degradation are deemed ineffective and are not recommended as candidates for use at Air Force sites.

5.3 Nutrient Data

The nutrient data are used to evaluate the background nutrient levels before amendment addition and the contribution to the nutrient pool from the amendment. The data provide information that is useful for evaluating enhancements observed in the condition containing the sterilized amendment that result from additional nutrients.

5.4 Oxygen Utilization and Carbon Dioxide Production Data

Respiratory data have been used to determine biodegradation rates in laboratory studies and in the field with applications such as bioventing. For the test described in this protocol, the data are used primarily for maintaining aerobic conditions and to indicate the rate of microbial activity in each reactor. Because the amendments often contain biodegradable components, respiration in the reactors could be the result of the degradation of these components and not the contaminant. Because of this, biodegradation rates based on either oxygen utilization or carbon dioxide production may overestimate the hydrocarbon degradation rate.

5.5 Petroleum Hydrocarbons in Soil Gas

The hydrocarbon data from the analysis of the gas collected during atmosphere exchanging are used to determine the mass of hydrocarbon removed from the soil through volatilization. The concentration data obtained from the GC analysis or the composited sample are converted to a mass of hydrocarbon removed by multiplying the concentration value by the volume of gas exchanged (300 mL). The cumulative mass is subtracted from the mass initially present in the soil to determine the amount of hydrocarbon available for biodegradation as described below.

5.6 Petroleum Hydrocarbons in Soil Samples

These are the most critical data with regards to evaluating any enhanced biodegradation due to the addition of the amendment. It is more appropriate to analyze the amendments on a mass-removed basis rather than on a reduction-in-concentration basis. To do this, the concentration data obtained from the GC analysis of the triplicate flasks are averaged and then converted to a mass of hydrocarbon present in the reactor. This is accomplished by multiplying the concentration data by the mass of soil (dry-weight basis) in each reactor.

To calculate the mass of hydrocarbon available for biodegradation, the mass of hydrocarbon removed during atmosphere exchanging is subtracted from the initial mass present in the soil. The mass remaining at the end of the incubation period is subtracted from the resulting initial values to determine the amount of hydrocarbon that was biodegraded in each reactor under each experimental condition.

6.0 REPORTING

The results from the tests described in this protocol are submitted in an evaluation report. The report will contain an introduction section that includes the microbial amendment identification and any pertinent vendor information. An experimental methods section is included that contains descriptions of the amendment preparation and usage procedures. In addition, any modifications made to the protocol described above are included along with an explanation of the need for that modification. The results section contains the results from the hydrocarbon analysis and presents the data in tabular form. The results from the remaining analyses are provided in an appendix. The discussion section focuses on the evaluation and interpretation of the data with emphasis on the performance of the amendment based on the hydrocarbon degradation. The final conclusion section contains the final evaluation of the amendment product. The report is submitted within 30 days following completion of the tests described in Section 4.0.

7.0 REFERENCE

Gardner, W.H. 1965. "Water Content." In C.A. Black, D.D. Evans, J.L. White, L.E. Ensminger, and F.E. Clark (Eds.), *Methods of Soil Analysis, Part 1 - Physical and Mineralogical Properties*, Including Statistics of Measurement and Sampling, American Society of Agronomy, Inc., Madison, WI. pp. 82-127.